Osteogenic Marker Expression in a Grafted Bone Healing Sheep Model

Anumala

A thesis submitted in partial fulfilment of requirements

for the degree of Doctor of Clinical Dentistry

University of Otago, Dunedin, New Zealand

August 2020

Abstract

Objectives: Successful healing of alveolar sockets after tooth extraction ensures positive outcomes for tooth replacement options. Using a sheep model the expression levels of key osteogenic markers for healing were compared over 16-weeks for empty and grafted sockets.

Methods and Materials: First, second and third premolars were extracted from 30 sheep. The socket was either non-grafted for spontaneous healing (control) or grafted using Bio-Oss® and Bio-Gide® (test). After 4-, 8- and 16-weeks the sheep were euthanised and tissue samples collected. Histological analysis was undertaken and cellular localisation of receptor RANK, and ligands RANKL and OPG was determined using immunohistochemistry. mRNA expression levels for RANK, RANKL, OPG, Col1A1, TIMP3, Sp7 and Msx2 were determined using SYBR green RT^2-qPCR assays.

Results: Overall, more new woven bone was present in the test group compared to the control at all time points. Moderate immunopositive staining of RANK was associated with osteoblasts and osteoclasts in both groups at 4 weeks; with stronger osteoclast-associated staining in the test group at 8- and 16-weeks. Strong staining of RANKL associated with osteoblasts and osteoclasts was found in both groups at all time points. Initial strong OPG staining localised to the connective tissues decreased over time. Similar levels of mRNA expression in both groups for all seven osteogenic genes was found. The exception was RANK with expression levels lower in the test group compared to the control group at 4-weeks (P = 0.02). Sp7 was also expressed significantly lower in test group at 16-weeks (P = 0.04).

Conclusion: Histologically more woven bone was present in test sockets likely due to the lower level of RANK expression resulting in decreased osteoclastic activity. There was, however, no statistically significant difference in the expression of the key markers of osteogenesis, RANKL and OPG between empty and grafted sockets at any time over the 16-week period. There was also no difference in the expression of the transcription factor for osteoblast differentiation MSX2 or the ECM markers Col1A1 or TIMP3. Although Bio-Oss® is not osteoinductive it has osteoconductive and scaffolding properties, playing a role in alveolar ridge preservation and therefore, its use should be continued.

Acknowledgement

I am thankful to God (ॐ श्री गणेशाय नमः) for providing me the strength, knowledge and guidance for making this thesis possible.

My sincere gratitude to Dr. Trudy Milne, my principal research supervisor for her patience, guidance, and support throughout this research project. I have learnt a lot about this topic and specific laboratory skills from Trudy. She has continuously motivated me to make this project possible. I would also like to thank my supervisors Associate Professor Dawn Coates and Professor Warwick Duncan. I have learnt a lot from Dawn and she has provided tremendous amount of assistance throughout. This project would not have been possible without Prof. Duncan who secured funding and carried out most of the surgeries and taught the surgical aspects of the research.

I would also like to thank my family; my mother Mrs. Daya Wati, my second set of parents Mrs. Shakuntla Devi and Mr. Dinesh Kumar and only my sister for their continuous encouragement and support.

A big thank you to AROA Biosurgery Ltd. for funding this project. Last but not the least, I would like to thank my classmates Drs. Saeideh Nobakht and Tatiana Tkatchenko for their assistance in this project. I would also like to extend my gratitude to my best friend Ramilta Singh, for being a close part of my journey and being my unpaid counsellor.

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List of Abbreviations

µg	Microgram
ARP	Alveolar ridge preservation
AWO	Animal welfare officer
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CBCT	Cone beam computed tomography
cm	Centimetre
Col1A1	Collagen, type I, alpha 1
CT	Connective tissue
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alii* (latin = and others)
H&E	Haematoxylin and eosin
IHC	Immunohistochemistry
i.m.	Intra-muscular
i.v.	Intra-venous
IgG	Immunoglobulin
kg	Kilogram
mm	Millimetre
Msx2	Homeobox protein MSX-2
NBF	Neutral buffered formalin
OPG	Osteoprotegerin
OV	Ovine
P1	First mandibular premolar
P2	Second mandibular premolar

P3	Third mandibular premolar
PBS	Phosphate buffered saline
PDL	Periodontal ligament
pH	Negative logarithm of hydrogen ion concentration
qRT^{2}PCR	Quantitative real time polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RNA	Ribonucleic acid
RT	Room temperature
Sp7	Sp7 Transcription Factor
TIMP3	Tissue Inhibitor of Metalloproteinases 3

Chapter 1 Introduction and Literature Review

1.1 Introduction

Loss of natural teeth is becoming increasingly prevalent. According to the 2009 National Oral Health survey, one in eleven (9.4%) adults in New Zealand have lost all their natural teeth (Ministry of Health, 2010). Tooth loss has negative impacts on masticatory function, speech and aesthetics and has recently been linked to cognitive decline (Nilsson et al., 2018).

Dental implants were first used for edentulous patients in an attempt to improve their quality of life by restoring function and aesthetics. By the late 1980s, implants were also being used in partially edentulous patients (Buser et al., 2017). With 11% of New Zealanders currently not having a functional dentition (Ministry of Health, 2010), the demand for a more permanent solution for restoring edentulous regions in the form of dental implants will only accelerate.

A number of human and animal studies have demonstrated the process of alveolar bone healing following tooth extractions (Amler, 1969; Cardaropoli et al., 2005; Scala et al., 2014; Trombelli et al., 2008). Up to 50%-dimensional changes can occur in the alveolar ridge during the healing of extractions socket in the premolar and molar regions (Schropp et al., 2003).

To compensate for the dimensional changes during the healing of extraction sockets and improve the success rates of dental implants, bovine-derived xenografts have been used in various animal models (Accorsi-Mendonça et al., 2008; Liu et al., 2016; Ramírez-Fernández et al., 2011; Tovar et al., 2014) and human trials (Barone et al., 2013). A commonly used xenograft, Bio-Oss® has been used in clinical practice, however, there are no published data which compares healing of the extraction socket in the presence of Bio-Oss® to natural bone healing. Hence, this study aimed to bridge this existing gap in knowledge with a study examining key osteogenic (bone-forming) markers at the molecular level.

1.2 Anatomy and Physiology of Bone

Bone can be categorised into four types: long, short, flat and irregular bones. The skull and mandible among other structures are made up of flat bones. Flat bones are made from membranous bone. Bones from varying skeletal regions contain different proportions of cortical and trabecular bone.

Cortical bone encloses the marrow space and is dense and solid. Trabecular bone comprises of a honeycomb like grid of trabecular plates and rods scattered in the bone marrow compartment. Cortical and trabecular bone are both made up of osteons.

Cortical osteons are known as Haversian systems and have a cylindrical shape. Each Haversian system is about 400 μm long and 200 μm wide and forms a diverging web within the cortical bone. Concentric lamellae shape the walls of the Haversian systems. The osteons in trabecular bone are known as packets and are made up of plates and rods that are 50 to 400 μm thick. Trabecular osteons are crescent shaped with a thickness of 35 μm and comprises of concentric lamellae.

A lamellar pattern is common for cortical and trabecular bone with collagen fibrils arranged in an alternating pattern. This alternating pattern gives lamellar bone its strength. However, this alternating pattern of collagen fibrils is not present in woven bone which makes it weaker than lamellar bone (Clarke, 2008).

1.2.1 Bone Growth, Modelling and Remodelling

Bone can undergo different forms of physiological change throughout life, such as longitudinal and radial growth, modelling and remodelling. Longitudinal and radial development takes place in the growth phase during childhood and adolescence. Longitudinal growth takes place at the epiphyseal and metaphyseal regions of long bone, which then undergoes mineralisation to form new primary bone.

Bone modelling involves changes in the overall shape of bone due to physiological or mechanical factors that cause slow alterations to the skeleton, a phenomenon described by Wolff's Law (Chen et al. 2010). Biomechanical forces can cause widening or alterations of axis by resorption or deposition of bone to suitable surfaces and this

process is mediated by osteoclasts and osteoblasts. In bone modelling, formation and resorption of bone are not tightly coupled.

Remodelling of bone involves bone renewal to preserve its strength and mineral balance. This process involves constant elimination of distinct packets of old bone that are replaced with new matrix, and mineralisation of the matrix results in new bone formation. Bone remodelling starts before birth and continues until death. Remodelling is a tightly coupled process involving the activity of osteoclasts and osteoblasts.

The cycle of remodelling can be described as four consecutive phases that includes activation, resorption, reversal and formation. The sites where remodelling takes place normally develop randomly, however, sites that need repair maybe be targeted (Burr, 2002; Parfitt, 2002). In the activation phase, mononuclear monocyte-macrophage osteoclast precursors are recruited and activated from the circulation. This causes the lining cells of the endosteum to detach from the bone surface and mononuclear cells to fuse together forming preosteoclasts. Preosteoclasts attach to bone matrix through integrin receptors in their cell membranes and RGD (arginine, glycine and asparagine) sequences, leading to formation of annular sealing zones surrounding a bone-resorbing compartment located below the multinucleated osteoclasts.

Each remodelling cycle that leads to bone resorption mediated by osteoclasts takes about two to four weeks. Formation, activation, and resorption is led by osteoclasts and controlled by the proportions of receptor activator of nuclear factor kappa-B ligand (RANKL) to osteoprotegerin (OPG), interleukin 1 (IL-1) and interleukin 6 (IL-6), colony stimulating factor (CSF), parathyroid hormone, 1,25-dihydroxyvitamin D and calcitonin (Blair & Athanasou, 2004; Boyle et al., 2003). Osteoclasts assist in mobilising bone mineral by releasing hydrogen ions into the resorption compartment that lowers its pH to approximately 4.5. Resorbing osteoclasts also release tartrate-resistant acid phosphatase, cathepsin K, matrix metalloproteinase 9, and gelatinase from cytoplasmic lysosomes that then form saucer-shaped Howship's lacunae on the surface of trabecular bone and Haversian canals in cortical bone by breaking down the organic matrix. Mononuclear cells complete the resorption phase as the multinucleated osteoclasts undergo apoptosis (Eriksen, 1986; Reddy, 2004).

Completion of bone formation takes about four to six months. Osteoblasts produce new collagenous organic matrix and control matrix mineralisation. Matrix mineralisation is achieved by release of small membrane-bound matrix vesicles that concentrate calcium and phosphate and enzymatically breakdown mineralisation inhibitors like pyrophosphate or proteoglycans (Anderson, 2003). Osteoblasts that are bounded by and submerged in the matrix, transform into osteocytes, contained within a space called a lacuna and maintained by cytoplasmic processes that extend from the osteocytes within canaliculi and connected to each other by gap junctions (Burger et al., 2003). The osteocyte network forms a functional syncytium within bone. A true syncytium results from fusion of multiple cells to a single multinucleated cell; the osteocyte network has a similar functional outcome, with each cell communicating by both intercellular gap-junctions and extracellular paracrine signalling and playing a role in mechanosensation, osteoclast mobilisation and regulation of phosphate homeostasis (Schaffler et al., 2014). When bone formation is completed, more than half of the osteoblasts undergo apoptosis, and the rest transform into osteocytes or bone-lining cells. Bone-lining cells may also control inflow and outflow of mineral ions from the extracellular fluid of bone. Hence, they act like a blood-bone barrier and have the capacity to transform back into osteoblasts post-exposure to parathyroid hormone or mechanical stimulation. Bone-lining cells in the endosteum lift off the bone surface prior to resorption to create distinct bone remodelling compartments that have specialised microenvironments (Clarke, 2008).

The product of every remodelling cycle is the production of a new osteon and the process is almost the same for cortical and trabecular bone. The remodelling units in trabecular bone are equal to cortical bone divided in half longitudinally. The difference between resorbed old bone and newly formed bone is known as bone balance. The bone balance for periosteum is slightly positive and for endosteum and trabecular bone it is slightly negative (Clarke, 2008).

The key functions of bone remodelling are conserving the mechanical strength of bone and maintaining healthy bone calcium and phosphate balance. Trabecular bone turnover rate is higher than that required for maintenance and this indicates higher turnover is essential for mineral metabolism. Increased bone remodelling units are required when

demand for calcium and phosphorus increases. This demand can also be met by increased activity of existing osteoclasts (Clarke, 2008).

1.2.2 Bone Cells: Osteoclasts, Osteoblasts, Osteocytes

1.2.2.1 Osteoclasts

Activated multinucleated osteoclasts are derivatives of mononuclear precursor cells of the macrophage lineage and are the only cells with the capacity to resorb bone (Boyle et al., 2003). The main source of mononuclear monocyte-macrophage precursor cells is bone marrow.

Two key cytokines that play an essential role in the formation of osteoclasts are RANKL and macrophage CSF (M-CSF). These cytokines are produced in both membrane-bound and soluble forms by marrow stromal cells and osteoblasts. The presence of stromal cells and osteoblasts in bone marrow is necessary for osteoclastogenesis. RANKL is part of the Tumour Necrosis Factor (TNF) superfamily and is essential in the formation of osteoclasts. M-CSF plays a role in proliferation, survival and differentiation of osteoclast precursors. M-CSF is also a key factor for survival of osteoclasts and maintaining the cytoskeletal rearrangement needed for bone resorption. OPG is a soluble protein and binds with RANKL to prevents its interaction with the RANK receptor (Cohen, 2006).

1.2.2.2 Osteoblasts

Osteoblasts arise from osteoprogenitor cells and maintain synthesis of new bone matrix. Osteocytes are entrapped within the bone matrix that supports bone structure. Subpopulations of cells from the osteoblast lineage react differently to different hormonal, mechanical or cytokine signals.

Pluripotent stem cells can self-renew and can produce osteoprogenitor cells in many tissues when the environmental conditions are favourable. There are a small number of mesenchymal stem cell populations that possess the capacity to make new bone, cartilage, fat or fibrous connective tissues; these are different to hematopoietic stem cells that produce the different lineages of blood cells (Pittenger et al., 1999).

Flattened bone-lining cells present on bone are dormant osteoblasts and are found in the endosteum on trabecular and endosteal surface and are present within the periosteum on the mineralised surface. Osteoblasts and lining cells are present close to each other and linked with adherens junctions. Cadherins are transmembrane proteins that hold cells together by linking their cytoskeletons with the help of tight junctions and desmosomes (Shin et al., 2000).

When preosteoblasts stop multiplying, osteoblast precursors alter their shape from spindle-shaped osteoprogenitors to large cuboidal differentiated osteoblasts on the surface of bone matrix. Preosteoblasts that are present near functioning osteoblasts, in bone remodelling units, are identifiable due to their expression of alkaline phosphatase (Clarke, 2008). Mature osteoblasts that are active produce bone matrix, have large nuclei, enlarged Golgi bodies and broad endoplasmic reticulum. These osteoblasts release type I collagen and other matrix proteins vectorially toward the bone surface.

Osteoblasts are diverse and possess the ability to express various repertoires. This heterogeneous nature is reflected in the differences in architecture at various sites within the skeletal system in health and disease and how osteoblasts respond treatment modalities for bone disease.

1.2.2.3 Osteocytes

Osteocytes terminally differentiate from osteoblasts and provide structural and metabolic support to bone. Osteocytes are found within the lacunae of mineralised bone and possess broad filipodial structures that are present in the canaliculi of mineralised bone. Typically, these cells do not express alkaline phosphatase, however, they do express osteocalcin, galectin 3 and CD44, a cell adhesion receptor for hyaluronate, and other matrix proteins that maintain intercellular adhesion and control exchange of mineral and fluid within the lacunae and canalicular network. During osteolysis, osteocytes are active and can carry out phagocytosis as they contain lysosomes.

Filipodial cellular processes help osteocytes retain associations with other osteocytes and the bone surface. Maintenance of gap junctions between cells and direct communications via intercellular channels are carried out by cellular proteins called

connexins. Metabolic and electrical communications of osteocytes are carried out via gap junctions that are made up of connexions (Plotkin et al., 2002). Gap junctions are crucial for function and survival of osteocytes.

The main function of the osteocyte-osteoblast/ lining cell syncytium is mechano-sensation (Rubin & Lanyon, 1987). Osteocytes transduce stress signals, from bending or stretching of bone, turning them into biological activity. Various activities in osteocytes are stimulated when canalicular fluid flows due to external forces. When bone calcium inflows rapidly across filipodial gap junctions, information is transferred among osteoblasts on the surface of the bone and osteocytes inside the bone. Prostaglandin E2, cyclo-oxygenase 2, various kinases, Runx2 and nitrous oxide are involved in the signalling mechanisms associated with mechano-transduction process.

Osteocytes can survive for decades in human bone without being involved in tissue turnover. Empty lacunae present in old bone indicate that osteocytes can go through apoptosis, and this is usually due to disturbance in their intercellular gap junctions or cell-matrix interactions (Xing & Boyce, 2005).

1.2.3 Extracellular Matrix of Bone

Protein in bone is made up of 85 to 90% collagenous proteins. Bone matrix is generally made up of type I collagen with minute amounts of types III and IV and FACIT collagens at different stages of bone formation that can assist in determining the diameter of the collagen fibrils. FACIT collagens are part of the Fibril-Associated Collagen family that have Interrupted Triple Helices; a group of nonfibrillar collagens that act as molecular bridges and are essential for the configuration and strength of extracellular matrices. Collagen IX, XII, XIV, XIX, XX, and XXI are members of this family. About 10 to 15% of the total proteins in bone are noncollagenous. About a quarter of noncollagenous proteins are derived exogenously, such as serum albumin and α2-HS-glycoprotein and due to their acidic characteristics, they can bind to hydroxyapatite. Serum-derived noncollagenous proteins can control matrix mineralisation and α2-HS-glycoproteins control bone cell proliferation. The remainder of the exogenously derived noncollagenous proteins include growth factors and other molecules that are present in minute quantities to regulate cellular functions.

Osteoblasts that produce and release noncollagenous proteins can be classified into various categories, however, the function of each bone protein is currently not well defined. Many proteins, however, have multiple functions such as controlling deposition and turnover of bone mineral and bone cell activity. Serum osteocalcin is derived both from matrix resorption via osteoclastic activity and osteoblast synthesis; it is considered a marker for bone turnover rather than a definitive marker of bone formation.

A key glycosylated protein present in bone is alkaline phosphatase, which is bound to the surface of osteoblasts through a phosphoibositol link and is present freely in mineralised matrix. Alkaline phosphatase has been proposed to promote extracellular bone mineralisation by inhibiting mineralisation inhibitor, inorganic pyrophosphate. Therefore, alkaline phosphatase regulates the mineralisation process (Halling Linder et al., 2017). The most common noncollagenous bone protein is osteonectin, which makes up about 2% of total protein in developing bone. Osteonectin is believed to have effects on osteoblast growth and/ or proliferation and mineralisation of matrix.

1.2.4 Bone Matrix Mineralisation

The composition of bone includes 50 to 70% mineral, 20 to 40% organic matrix, 5 to 10% water and less than 3% lipids. The major mineral component of bone is hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], with small quantities of carbonate, magnesium and acid phosphate and hydroxyl groups (Clarke, 2008).

Expression of alkaline phosphatase and various noncollagenous proteins such as osteocalcin, osteopontin and bone sialoprotein have been associated with matrix maturation. Calcium and phosphate-binding proteins are associated with controlling deposition of mineral via regulation of hydroxyapatite quantity and size.

1.3 Alveolar Process Anatomy

The alveolar process can be described as bony tissues that surround a fully erupted tooth and are in accord with teeth development and eruption. Coronally it is bordered by the bone margins of the socket. An imaginary line crosses the base of the socket perpendicularly along the long axis of the root. The basal bone of the mandible or maxilla is located to apical this line (Araujo et al., 2015).

The morphological features of the alveolar process are associated with the size and shape of the tooth, tooth eruption site, and gradient of tooth eruption. The gradient and eruption of teeth is normally away from the basal bone centre (Pietrokovski & Massler, 1967).

The inner aspects of the socket walls are known as the alveolar bone proper or bundle bone, and the rest of the hard structure is termed alveolar bone. Bundle bone is lamella bone and is made up of circumferential lamellae with a width of 0.2 to 0.4 millimetres (mm). Alveolar bone is also made up of lamellar bone, specifically the concentric and interstitial type and bone marrow. The Sharpey's fibres in bundle bone are arranged in a fashion that joins the periodontal ligament to the alveolar bone and skeleton. On the opposite side of the periodontal ligament, the Sharpey's fibres are inserted into the cementum which attaches the periodontal ligaments to the dentin. Bundle bone is dependent on the tooth structure and is relationship to the cementum and periodontal ligament on the tooth roots (Araujo et al., 2015).

1.3.1 Socket Healing

The healing process of an alveolar socket can be described as changes that occur in the dimensions of the socket and those that occur histologically.

1.3.1.1 Dimensional Changes

Multiple human studies have reported changes in dimensions in the alveolar socket post tooth extraction (Atwood, 1963; Bergman & Carlsson, 1985; Johnson, 1963, 1969; Pietrokovski et al., 2007; Schropp et al., 2003; Trombelli et al., 2008). Significant vertical and horizontal contractions occur in the alveolar ridge following multiple tooth

extractions (Carlsson & Persson, 1967; Johnson, 1969). For individuals wearing full dentures for several years, the resorption in the alveolar ridge exhibit wide variations (Carlsson et al., 1967). Extraction of a single tooth results in more limited dimensional changes vertically, with significant changes occurring horizontally (Pietrokovski & Massler, 1967; Pietrokovski et al., 2007). The ridge width reduces almost by 50% with greater resorption occurring on the buccal aspect in comparison to the lingual/ palatal aspect, with more resorption occurring in the molar regions.

The completion of socket healing is clinically defined as when the opening of the socket is closed by firm epithelialised soft tissues and/ or radiographically the socket has been filled with of bone. The time taken for complete socket healing varies among individuals (Carlsson & Persson, 1967; Johnson, 1963, 1969; Schropp et al., 2003; Trombelli et al., 2008). Closure of the socket opening takes about 10 to 20 weeks (Johnson, 1969; National Research Council, 1980) with radiographic evidence of bone fill ranging from three to six months (Schropp et al., 2003). Most of the dimensional changes occur within the first three months after extraction, however, restructuring of the alveolar ridge lasts for about 1 year (Brkovic et al., 2012; Schropp et al., 2003).

1.3.1.2 Histological Changes

Various studies on humans and dogs have been carried out to demonstrate the events of socket healing (Amler, 1969; Araujo & Lindhe, 2005, 2009a; Araujo et al., 2005; Araujo et al., 2006a, b; Carlsson & Persson, 1967; Trombelli et al., 2008). The process of socket healing, post extraction, can be described as three sequential and interrelated phases: inflammatory, proliferative and modelling/ remodelling.

The inflammatory phase can be further divided into two sub-phases: formation of a blood clot and migration of inflammatory cells. The tooth socket fills up with blood immediately after extraction forming a platelet plug. In the next 2-3 days inflammatory cells migrate to cleanse the wound prior to new tissue formation. Inflammatory cells, vascular sprouts and immature fibroblasts combine to form granulation tissue. The granulation tissue slowly gets replaced with a temporary connective tissue matrix and the proliferative phase starts.

The characteristic feature of the proliferative phase is rapid tissue formation. This phase is also further divided into two, fibroplasia and establishment of woven bone. A provisional matrix is formed during fibroplasia. This temporary matrix eventually gets infiltrated with vessels and osteoblasts giving it a finger-like appearance within woven bone surrounded by blood vessels. When the projections fully surround a vessel, it gives rise to the primary osteons. Woven bone becomes identifiable in the socket approximately two weeks after extraction and can last for numerous weeks. Since woven bone lacks load-withstanding abilities it is essential that mature bone (lamellar bone and bone marrow) supplants it.

The third and last phase of socket-healing is bone modelling and remodelling. Bone modelling involves alterations in the shape and structure of bone. Remodelling involves changes without associated alterations in shape and structure. In the remodelling process the lamellar bone or bone marrow replaces woven bone. In bone modelling, dimensional changes of the alveolar ridge occur due to the resorption of socket wall. In humans bone remodelling can last for about several months however, a lot of individual variability exists (Carlsson & Persson, 1967; Trombelli et al., 2008).

Biopsies from socket sites in the posterior maxilla after more than 16 weeks of healing showed that lamellar bone and bone marrow make up 60-65% of tissue volume (Lindhe et al., 2012). Therefore, completion of woven bone remodelling into lamellar bone and bone marrow can take up to several months to years. Resorption of socket walls in humans (Carlsson et al., 1967) and dogs (Araujo et al., 2009, 2008) has been associated with the presence of osteoclasts surrounding the crest of buccal and lingual walls and on the exterior and interior (bundle bone) aspects of the socket. Equal amounts of bone modelling take place on the lingual and buccal bone, however, because the buccal bone wall is thinner than the lingual wall, modelling causes more vertical bone loss. Bone modelling occurs earlier than bone remodelling, such that more than half of the modelling takes place within the first three months of healing (Schropp et al., 2003). Remodelling and modelling processes result in reductions to the size of the alveolar ridge.

1.4 Bone Healing

Bone healing with grafting materials (bone or bone substitutes) can take place via osteogenesis, osteoconduction or osteoinduction. On the other hand, bone healing around dental implants occurs through osseointegration.

1.4.1 Osteogenesis

This process involves growth of new bone via osteoblasts that originate from the grafting materials, such as when autologous grafts are used. Osteogenic grafts, such as autografts contribute to new bone formation due to presence of osteoblasts or cells that transform into osteoblasts. The grafts are also a source of growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF) and vascular endothelial growth factor (VEGF) that regulate osteogenesis and angiogenesis (Wang & Yeung, 2017).

1.4.2 Osteoinduction

This term describes the process where undifferentiated and pluripotent cells are triggered to develop into a bone-forming cell lineage (Albrektsson & Johansson, 2001). Undifferentiated cells can be recruited to form osteoprogenitor cells (Young, 1963) that will differentiate into bone cells, and therefore are essential for adequate bone healing or anchorage of implants. This requires an adequate trigger (inductive agents), and the presence of undifferentiated mesenchymal cells, which are then differentiate into preosteoblasts which mature and lay down new bone.

Classic research describing bone induction at different sites in a host, used gall bladder epithelium, alcohol extracts of bone, and transplants to muscles or the anterior chamber of the eye to show heterotopic bone formation (Huggins, 1968; Levander, 1938; Urist & McLean, 1952). Historically the most reliable way to determine the osteoinductive properties of a material was to inject it into a heterotopic bed, like a muscle pouch, and to examine bone formation. Inductive agents naturally function around bone as well; however, it is difficult to differentiate between bone induction and conduction in an orthotropic site.

In later research, Urist et al. (1979) isolated bone morphogenic proteins (BMPs), which are soluble glycoproteins proven to act as inductive bone agents. BMPs are part of the transforming growth factor (TGF)-β-family and there are at least 15 identified BMPs, with BMP-2 and BMP-7 of most interest during osteogenesis. Trauma or bone remodelling can naturally trigger release of BMPs, as the most recognised bone inductive agents. Bone induction can also be directly or indirectly triggered by physical stimuli like stress or various electrical signals (Bassett, 1968; Buch, 1985; Yasuda, 1953).

Graft materials are considered osteoinductive when bone formation is stimulated in soft tissues next to the graft material.

1.4.3 Osteoconduction

Osteoconduction is a term used to describe bone growth on a surface. A surface is considered osteoconductive when it allows bone growth on its surface or down into its pores or channels. Osteoconduction is a process where bone is absorbed to adapt onto the surface of a material. However, this definition is restricted as it has little or no association to biomaterials (Wilson-Hench, 1987).

In practice, osteoconduction is dependent on prior osteoinduction. Many different factors are essential for bone formation. Albrektsson (1980), examined *in vivo* bone conduction and remodelling and reported that full vascularisation was important for formation of bone (Albrektsson, 1980). Thus, if an adequate blood supply is absent, bone growth and bone conduction does not take place. It has also been noted that the key role of many growth factors is thought to be mitogenic and angiogenic (Trippel et al., 1996). Growth factors regulating bone regeneration include insulin-like growth factor (IGF I, II), fibroblast growth factor (FGF), TGF-β and platelet-derived growth factor (PDGF) (Albrektsson & Johansson, 2001).

Thus, osteoconductive biomaterials do not contribute to new bone formation but serves as a scaffold for bone formation that originates from adjacent host bone. Some examples of osteoconductive biomaterials include xenografts like Bio-Oss®, freeze dried bone allograft (FDBA) and calcium phosphate.

1.4.4 Osseointegration

The term osseointegration was initially described by (Branemark et al., 1977) and (Albrektsson et al., 1981), who used light microscopy and described direct contact between live bone and implant. From a biomechanical perspective it has been described as an asymptomatic, firm fixture of alloplastic materials that are attained and retained in bone during functional loading (Zarb & Albrektsson, 1991). Even though osseointegration depends on osteoinduction and osteoconduction, the description of the term indicates that anchorage of bone is sustained over time.

1.5 Osteogenic Markers of Bone Healing

For bone to adequately function, it is important to maintain a balance between osteoblasts and osteoclasts. Osteoblasts are activated and their differentiation is controlled by a number of signalling proteins such as Wnt signalling pathways, Indian Hedgehog and Runt-related transcription factor 2 (Runx2). In addition to these signalling proteins, osteoblast differentiation and activation is also regulated by different transcription factors and genes as described below.

1.5.1 Tumour Necrosis Factor Receptor Superfamily Member 11A (TNFRSF11A / RANK)

Receptor activator of nuclear factor kappa B (RANK) is also known as TNF-related activation-induced cytokine (TRANCE) receptor or tissues necrosis factor receptor superfamily member 11A (TNFRSF11A). RANK was first identified by Anderson and colleagues (1997) as a receptor for RANKL. RANK is a type I transmembrane protein and belong to the tumour necrosis factor receptor (TNFR) family. RANK is made up of 616 amino acids with four extracellular cysteine-rich pseudo-repeat domains (CRDs) (Anderson et al., 1997). Skeletal muscle, thymus, liver, large intestine, small intestine, adrenal glands, osteoclasts, epithelial cells in mammary glands, vascular cells and pancreas all express RANK (Sattler et al., 2004).

RANK is expressed on osteoclasts and their precursors and is activated by its ligand RANKL. This interaction between RANK and RANKL stimulates formation and activation of osteoclasts and also increases survival of osteoclasts by reducing apoptosis (Walsh et al., 2013b). OPG competes with RANKL and controls activation of the RANK signaling pathway, thus OPG acts a decoy receptor for RANK. Various signaling pathways are involved in the differentiation and activation of osteoclasts by RANK.

The effects of RANK on osteoclasts has been was demonstrated in a knockout study in mice. RANK deficient mice have shown to exhibit severe osteopetrosis attributed to inhibition of osteoclast formation (Dougall et al., 1999). This observation is based on the findings that RANK is expressed on hematopoietic cells and osteoclasts, and not on osteoblasts and stromal cells (Jimi et al., 1999). Therefore, it is apparent that a lack of

osteoclast differentiation observed in RANK deficient mice is related to an inherent defect in the hematopoietic lineage.

Tumour necrosis factor receptor (TNFR) associated factor 6 (TRAF-6) belongs to the tumour necrosis factor (TNF). Five members of the TRAF family interact with RANK, however, and TRAF-6 has been an essential adaptor molecule for RANK and regulator for osteoclasts formation (Darnay et al., 2007; Lamothe et al., 2007). A study of TRAF-6 deficient mice found that they exhibited osteopetrosis lesions, impaired bone remodelling and tooth eruption. The authors reported that these impairments were caused by diminished osteoclast function (Lomaga et al., 1999). A possible mechanism for this is that TRAF-6 can transduce RANK signalling, therefore acting as a regulator for osteoclast activation (Darnay et al., 2007; Lomaga et al., 1999). In summary, these studies provided evidence on the role of RANK in formation and activation of osteoclasts.

1.5.2 Tumour Necrosis Factor Superfamily Member 11 (TNFSF11/ RANKL)

Tumour Necrosis Factor Superfamily Member 11 (TNFSF11/RANKL) was discovered to have a role in bone remodelling when it bound an OPG probe and was thus called both OPG ligand (OPGL) and osteoclast differentiation factor. OPGL showed similarity to two earlier identified molecules, namely TNF-related activation induced cytokine (TRANCE) and receptor activation of NF-κB ligand (RANKL) (Theoleyre et al., 2004). Ikeda and colleagues (2001) described three isoforms of RANKL. The RANKL1 and RANKL2 isoforms have transmembrane domains with the ability to form complexes on cell membranes, made of multiple monomers (Ikeda et al., 2001). RANKL3 is soluble and does not have a transmembrane protein domain.

RANKL is a glycoprotein that belongs to the TNF family. RANKL is also recognised by other terminologies such as tumour necrosis factor ligand superfamily member 11 (TNFSF11), TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) and osteoclast differentiation factor (ODF). Osteoblasts, stromal cells and T cells can express RANKL (Arabaci et al., 2015). RANKL expression can take place in three distinct molecular varieties and can consist of a trimeric transmembrane protein, primary secreted form or truncated ectodomain (Findlay & Atkins, 2011). Proteases such as matrix metalloproteinases (MMPs), disintegrin and metalloproteinase domains

(ADAMs) can also cleave RANKL (Georges et al., 2009). A total of 314 amino acids make up RANKL and it has five exons in its gene sequence (O'Brien, 2010; Walsh et al., 2013a).

RANKL binds with a homotrimeric disulfide-linked form of RANK onto the surface of cells (Théoleyre et al., 2006). These interactions between RANK and RANKL activate various signaling pathways that regulate the differentiation, activation and survival of osteoclasts and hence regulate resorption of bone (Boyle et al., 2003; Lacey et al., 1998). Kong and colleagues (1999) reported that RANKL-deficient mice exhibited severe osteopetrosis and defective tooth eruption defects. This was attributed to the diminished capability of osteoclasts to carry out osteoclastogenesis, as RANKL deficient mice demonstrated a total absence of osteoclasts (Kong et al., 1999).

1.5.3 Tumour Necrosis Factor Receptor Superfamily Member 11B (TNFRSF11B/ OPG)

The Amgen group was the first to discover osteoprotegerin (OPG) and found that increased expression of OPG was associated with osteopetrosis in transgenic mice (Simonet et al., 1997). This study demonstrated that OPG regulates bone density and negatively regulates osteoclast maturation. Therefore, increased expression of OPG leads to increased bone mass (osteopetrosis) due to a lack of osteoclasts and imbalances between osteoblast and osteoclast functions (Simonet et al., 1997).

OPG is also known as osteoclastogenesis inhibitory factor (OCIF) and tumor necrosis factor receptor superfamily member 11B (TNFSF11B). OPG is a soluble glycoprotein that belongs to the TNF-receptor superfamily. OPG can be present either as a 6-kDa monomer or a 120-kDa dimer when joined by two sulphite bonds (Baud'huin et al., 2013). A total of 380 amino acids make up OPG proteins that create seven functional domains. The first four domains are present on the cysteine-rich N-terminal and bind with RANKL. The fifth and sixth domains are the death domains that are involved in OPG dimerisation. The last domain is known as the C-terminal heparin-binding domain that ends with a cysteine (Cys-400) and this domain has a key role in dimerisation of OPG (Schneeweis et al., 2005).

OPG is secreted by stromal cells and osteoblasts lineage cells. OPG acts as a decoy receptor for RANKL, counteracting its role in formation of osteoclasts. In addition to

osteoblasts, OPG is expressed by numerous tissues such as heart, kidney, liver, spleen and bone marrow (Wada et al., 2006). Overall, OPG prevents osteoclast activation and stimulates apoptosis. The proportions of RANKL and OPG maintain bone balance.

Simonet and colleagues (1997) reported that overexpression of OPG in mice resulted in profound yet nonlethal osteopetrosis and diminished differentiation of osteoclasts. This study demonstrated that OPG plays an essential role in the maintenance of bone mass (Simonet et al., 1997). Confirming this finding was a mice study where OPG was depleted, there was decreased density of bone and marked porosity was observed (Bucay et al., 1998). In another study on OPG deficient mice, increased osteoclastogenesis was reported that resulted in severe osteoporosis. Reduced mechanical strength of bone accompanied by bone loss was also reported in OPG deficient mice. The authors reported that OPG can negatively regulate osteoclastogenesis (Mizuno et al., 1998). These results indicate that OPG is essential in regulating bone mass.

1.5.3.1 The RANK/ RANKL and OPG Axis

The RANK/ RANKL/ OPG axis has multiple physiological roles such as maintaining bone homeostasis, immunity and osteoimmunology (Walsh & Choi, 2014). The axis has an important role in pathologies such as inherited and acquired bone pathologies. This section will focus on the role of the RANK/ RANKL/ OPG axis on bone homeostasis and bone healing.

The role of the RANK/ RANKL/ OPG axis plays in bone remodelling has been well established with transgenic and gene-knockout studies (Wright et al., 2009). Osteocytes play a key role in coordinating bone remodelling in healthy bone by regulating osteoblasts and osteoclasts via RANKL (Nakashima et al., 2011). Many small fractures occur in the skeleton that can trigger bone remodelling (Boyce & Xing, 2007; Verborgt et al., 2000). Osteocytes identify these fractures and inhibit osteogenesis by releasing sclerostin which is a by-product for osteocyte apoptosis (Colopy et al., 2004; Fazzalari, 2011; Verborgt et al., 2000). The surrounding osteocytes respond to this signal and release growth factors and cytokines which stimulate bone marrow-derived mesenchymal stem or stromal cells. The MSCs in turn divide and differentiate into pre-osteoblasts. The pre-osteoclasts are responsible for releasing Macrophage Colony-

Stimulating Factor (M-CSF) and expressing RANKL (Sharaf-Eldin et al., 2016). These trigger colony-forming unit -macrophages (CFU-M) to turn into pre-osteoclasts that merge into multinucleated osteoclasts with RANK receptors. The evidence thus suggests that the first stage in bone remodelling and healing is inflammation.

Activation of osteoclasts can occur when they bind RANKL, either in its soluble state or when RANKL is bound to the membrane on pre-osteoblasts (Sharaf-Eldin et al., 2016; Théoleyre et al., 2006). Osteoclasts release hydrochloric acid and hydrolytic enzymes that break down injured or broken bone. This process triggers release of growth factors such as transforming growth factors (TGF) and BMPs from the bone matrix which then drive the formation of MSCs into osteoblasts. The osteoblasts ultimately surround the resorbed pit, filling it with osteoid that later turns into woven bone (Boyce & Xing, 2008; Fazzalari, 2011). At the same time OPG is released by osteoblasts and connective tissue cells. OPG attaches to RANKL leading to the apoptosis of osteoclasts and hence decreases osteoclastogenesis (Figure 1). Towards the end of the remodelling cycle woven bone is remineralised by osteoblasts. The osteoblasts ultimately either undergo apoptosis or transform into osteocytes (Kapasa et al., 2017). Overall, a higher ratio of RANKL/ OPG results in more bone resorption and a higher OPG/ RANKL ratio favours more bone formation (Kapasa et al., 2017).

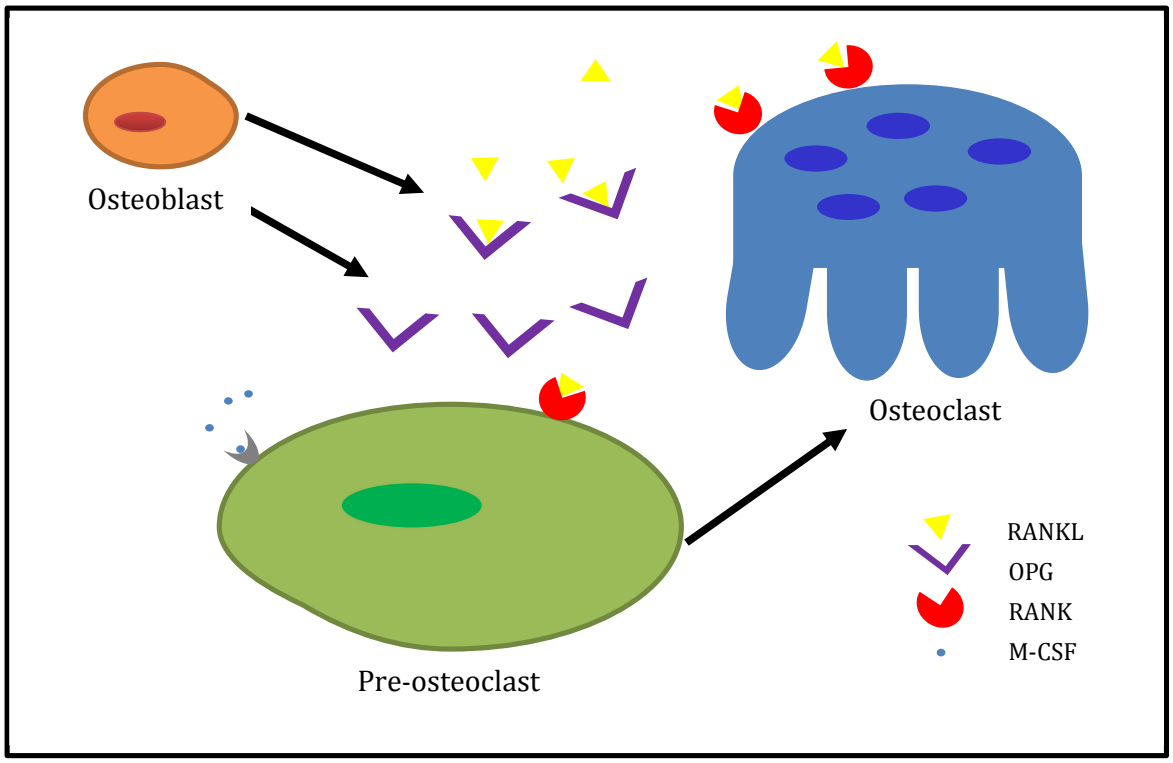


Figure 1: OPG/ RANKL pathway.
Adapted from (Castrogiovanni et al., 2016)

When the ratio of RANKL and OPG are comparable, bone remodelling is considered to be stable. During bone healing, the expressions of RANKL and OPG changes in a temporal manner. In an alveolar healing model in rats, Hassumi and colleagues (2018) investigated expression of RANKL and OPG after extraction of incisor teeth at 7, 14 and 28 days. The IHC results in the study showed no difference in the expression level of RANKL and OPG, as both showed moderate staining over the three healing points. RANKL was expressed mostly in osteoblasts and fibroblasts in the trabecular bone in the early healing process and in osteocytes in newly formed trabecular bone in the late healing phases. The expression of OPG was observed in connective tissue in the early phases of healing and in fibroblasts and osteoblasts around the edges of the wound. The mRNA expression of OPG increased in the later healing period whereas the expression of RANKL peaked during the 14-day time point and was low in the early and late phases (Hassumi et al., 2018).

In a study, van Hout et al. (2015) compared the osteogenic characteristics of Biosilicate® and Bio-Oss® in osteoporotic lesions in ovariectomised rats and found increased

expressions of OPG and RANKL in the osteoporotic lesions treated with Biosilicate®. There was no difference in the expression of genes in osteoporotic lesions filled with Bio-Oss® and healthy controls (van Houdt et al., 2015).

A larger animal sheep model was used by Baharuddin and colleagues (2015) to assess the regeneration of bone into periodontal wounds compared to unwounded furcation defects at six hours and one, four- and six-weeks post-surgery. The study evaluated healing and RANK, RANKL and OPG using IHC at three time point over six weeks. The study showed the presence of OPG only in unwounded tissues. RANK, RANKL and OPG were all expressed in wounded tissues as healing progressed. Strong expression of RANKL was present in the later phases (six weeks) of healing and this was associated with osteoclasts and osteoblasts close to the wounded bone. Expression of RANK was low in the early phases of healing; however, strong staining was observed in osteoclasts around the wound edges in the late phase of healing. Expression of OPG was associated mostly with the connective tissues and its expression consistently increased over the healing period (Baharuddin et al., 2015).

The randomised controlled trial conducted by Mandarino et al. investigated with one group with natural healing and the other group after receiving a dense polytetrafluoroethylene membrane. Over a healing period of four months, the mRNA expression for RANKL and OPG was similar in gingival fibroblasts and osteoblasts. Histological assessments showed the presence of osteoblasts near the osteoid tissues. Woven and mature bone were present in trabecular bone with an abundance of osteocytes and minimal osteoblastic activity (Mandarino et al., 2018).

Expression of RANK, RANKL and OPG has also been evaluated in natural healing and grafted sockets in three treatment groups in humans (Canciani et al., 2017). The treatment groups included no grafts, sockets filled with a scaffold and sockets filled with a granular grafting material. The authors reported that the expression of all three genes increased over the healing period of four months. The gene expressions ratio for RANKL to OPG was negative for empty sockets and sockets filled with scaffold. The sockets filled with the granular grafting material showed a positive RANKL/ OPG ratio. Overall, even though the empty sockets and granular grafted sockets were similar in terms of healing, the gene expression revealed slower healing in the granular grafted group.

A histomorphometric and gene analysis study investigated natural healing against alveolar sockets grafted with magnesium-enriched hydroxyapatite (MgHA), immediately after extraction and four months post healing. The expression of RANK was observed in connective surrounding mature bone and was especially concentrated near the grafting material. RANKL showed mild staining in the extracellular space inside the medullary space, whereas OPG appeared in the medullary space during mineralisation. OPG staining was more apparent within the connective tissues at the remodelling front and within the Haversian canals. A positive RANKL/ OPG ratio was present over the four-month healing period (Canullo et al., 2016).

1.5.4 msh Homeobox 2 (Msx2)

Msx2 belongs to the *Drosophila msh* gene family and is also known as Hox 8. This gene is a transcription factor that regulates activities of the other genes and is located on distinct sections of DNA. Msx2 is expressed in different tissues at various stages of growth and development (Davidson, 1995). Hodgkinson and colleagues (1993) reported that expression and regulation of Msx2 is controlled by vitamin D3 in human osteoblasts. Vitamin D3 has been linked to the regulation of proliferation, maturation, differentiation and apoptosis of the bone cells (Hodgkinson et al., 1993). The Msx2 protein carries out its functions via the bone morphogenetic protein pathway (Ferrari et al., 1998; Vainio et al., 1993).

Liu and colleagues (1999) used a mice calvarial model and found that an increased number of osteogenic cell when Msx2 was overexpressed. The overexpression of Msx2 was associated with osteoblasts and resulted in the increased growth of the parietal bone (Liu et al., 1999). Similar findings were reported in a chick calvarial model (Dodig et al., 1999). Deficiencies of Msx2 in mice have been reported to be associated with impaired bone and calvarial formation, chondrogenesis impairments, diminished osteoblasts and ectodermal organs defects including teeth, hair and mammary glands (Satokata et al., 2000).

Studies on mice models have reported that Msx2 plays a positive role in the growth and development of bone. On the other hand, genetic *in vitro* studies have demonstrated that transcription of osteoblastic-specific genes like osteocalcin can be negatively regulated

by Msx2 (Ryoo et al., 1997; Towler et al., 1994). Msx2 also has the ability to hinder transcription of Runx2, which is an essential transcription factor in differentiation of osteoblasts (Shirakabe et al., 2001). Collectively these studies demonstrate that Msx2 negatively regulates maturation of osteoblasts.

In another mice model, Ichida and colleagues (2004) examined the effects of Msx2 on osteoblast differentiation of mesenchymal cells. The authors reported that Msx2 stimulates the differentiation of mesenchymal cells into an osteoblast lineage without depending on Runx2 (Ichida et al., 2004). Therefore, Msx2 has been shown to control transcription and differentiation of mesenchymal cells into osteoblasts that could impact bone healing.

1.5.5 Sp7 Transcription Factor (Sp7)/ Osterix/Osx

Sp7 is also known as osterix or osx and is a member of the Sp family. It is a zinc-finger transcription factor (Peng et al., 2013), and has been shown to be required during the development and mineralisation of bone (Lee et al., 2003; Nakashima et al., 2002; Nishio et al., 2006). All endochondral and membranous bone osteoblasts express Sp7 (Sun et al., 2008). Expression of osteoblastic genes such as osteocalcin (OCN), osteopontin (OPN), collagen type I (COL1) and bone sialoprotein BSP) are also controlled by Sp7 (Sun et al., 2008).

Different mice studies have demonstrated that Sp7 has an important role in differentiation of osteoblasts and bone formation. It has been reported that Sp7 deficient mice exhibit impaired bone formation (Baek et al., 2009, 2010; Nakashima et al., 2002; Zhou et al., 2010). Yoshida and colleagues (2012) reported an increased Sp7 expression during the early phases of osteoblast differentiation in mice. However, Sp7 also inhibited differentiation of osteoblasts in the later (Yoshida et al., 2012). This auto-regulation of Sp7 expression that is dependent on stage of osteoblast differentiation plays a vital role in formation and maintenance of bone.

Apart from Sp7 expression in the development and maintenance of bone, its role in bone healing and regeneration has also been explored. Tu and colleagues (2007) carried in a mice calvarial study to evaluate the overexpression of Sp7 in bone marrow-derived

mesenchymal stem cells (BMSCs) during bone regeneration. The authors reported that 85% of the defects with overexpression of Sp7 transduced, healed and had five times more new bone formation compared to the group with empty vector transduction (Tu et al., 2007). In other mice model studies, it has been shown that BMSCs that express Sp7 had bone formation that increased osseointegration around dental implants (Tu et al., 2006; Xu et al., 2009).

In summary, it has been well documented in small animal models that Sp7 plays an essential role in bone development. This is achieved by regulation of differentiation osteoblasts, and therefore, Sp7 may also play a vital role in bone healing.

1.5.6 Collagen Type I alpha 1 chain (COL1A1)

Type I collagen is the most common type of collagen in humans. Collagens provide strength and support to various types of tissues including cartilage, bone, tendon, skin and sclera. Type I collagen consists of three chains, two alpha-1 chains and one alpha-two chain (Kuivaniemi et al., 1991). The two collagen alpha-1 chains are the key organisational proteins of bone, teeth and tendons (Viguet-Carrin et al., 2006). Reduced expression or mutations of the Col1A1 gene can therefore, result in compromised bone healing due to alterations in formation of collagen fibers (Stover & Verrelli, 2011).

A mouse model was used to evaluate alveolar socket healing post extraction at zero hours and after 7, 14 and 21 days (Vieira et al., 2015). It was reported that Col1A1 expression was elevated during bone healing compared to controls without extractions. Peak levels of Col1A1 were observed on the seventh day, with steady reduction after that time point. It has been demonstrated that Col1A1 is a marker of early bone healing and hence is highly expressed in the early phases of healing (Berendsen et al., 2014; Cardoso et al., 2011; Stover & Verrelli, 2011). A similar temporal pattern has also been reported for bone healing around oral implants (Biguetti et al., 2018).

An ovine model has been used to evaluate the mRNA expression of genes during normal versus hindered fracture healing over different time periods (Lienau et al., 2010). The authors reported that Col1A1 expression increased over the healing periods for both groups; however, there were significantly lower expressions in the delayed fracture

healing group. This suggests that expression of Col1A1 is reduced during compromised bone healing.

In summary, studies have demonstrated that Col1A1 expression is a marker of extracellular matrix production essential for adequate bone healing especially during the early phases of repair and regeneration.

1.5.7 Tissue Inhibitor of Metalloproteinases 3 (TIMP3)

TIMP3 is part of the tissue inhibitor of metalloproteinases (TIMPs) family of genes. This group of genes are responsible for supressing matrix metalloproteinases responsible for breakdown the extracellular matrix (ECM). TIMP3 has certain characteristics that make it unique when compared to other TIMP family members. TIMP3 is closely attached to the ECM, as initially discovered through the presence of TIMP3 in the ECM of fibroblasts in the chicken embryo (Blenis & Hawkes, 1984). TIMP3 has the potential to suppress ADAMs (A Disintegrin and Metalloproteinases), such as tumour necrosis factor alpha (TNF-α) converting enzyme (Amour et al., 1998). This may explain how TIMP3 can facilitate apoptosis of tumour cells (Smith et al., 1997) and hinder detachment of L-selectin and interleukin-6 (IL-6) receptors (Borland et al., 1999; Hargreaves et al., 1998).

A pig model was used to demonstrated that TIMP3 can reduce breakdown of cartilage as evidenced by a reduction in apoptosis of chondrocytes (Gendron et al., 2003). A study in mice reported that TIMP3 deficient mice developed osteoarthritis at a faster rate compared to controls (Clements et al., 2003). Various studies have reported that that TIMP3 expression is greater in tissues that undergo massive remodelling especially during embryonic and post-natal phases and in tissues with high turnover rates (Poulet et al., 2016). An ovine model evaluating mRNA expression of genes during hindered fracture healing (Lienau et al., 2010), demonstrated that there was no increased expression of TIMP3 when delayed healing was induced. The results of the study suggest that the level of MMPs and its inhibitors remains stable for adequate healing of induced delayed fracture wounds.

TIMP3 also plays a critical role in bone development affecting both the amount and properties of bone. Poulet and colleagues (2016) used a mice model to demonstrate that

when TIMP3 is overexpressed it can disrupt endochondral development especially during the early stages. During the post-natal and adult phases TIMP3 overexpression alters the structure and function of bone. The study also reported that temporary TIMP3 transgene expression can also impact on the function of osteoblasts in the chondrocyte-to-osteoblast lineage that in turn have persistent alterations in bone.

TIMP3 also has anti-angiogenic potential. It inhibits release of angiogenic components, therefore, hinders blood vessel invasion in the ECM (Anand-Apte et al., 1997). Biasutti and colleagues (2017) reported that elevated TIMP3 expression in post-surgical tendon wounds limited formation for new blood vessels after injury and during the healing process (Biasutti et al., 2017).

A systematic review that evaluated the available evidence on the effects of gene expression in early healing of dental implants in humans, reported that TIMP3 was identified to be involved in osteoclastic and remodelling activities (Shanbhag et al., 2015). To summarise, evidence suggests that TIMP3 plays a critical role in regulating ECM turnover in numerous types of tissues and hence also has a key role in wound healing.

1.6 Alveolar Ridge Preservation

Spontaneous healing following extraction of teeth results in dimensional changes of the socket walls. A systematic review (Tan et al., 2012) examined 20 different human studies and reported that reduction in the vertical direction ranged from 11-22% (-1.24 ± 0.11mm) and in the horizontal direction from 29-63% (-3.79 ± 0.23mm). A reduction of bone contour of 0.5-1% per year can be expected (Ashman et al., 1994). For single tooth extractions, up to 50% reduction in alveolar ridge width can take place, especially on the buccal aspect (Araujo et al., 2015).

These dimensional changes in the extraction socket can be minimised and bone deposition can be enhanced using bone grafts, bone substitute grafts or membranes, either alone or in combination. This procedure is known as alveolar ridge preservation. There are certain clinical scenarios where alveolar ridge preservation may not be feasible, for instance in the presence of acute infection. In these circumstances, ridge preservation can be delayed by six to eight weeks post-extraction (Darby et al., 2008). In a consensus report, Chen and colleagues (2004) reported that minimal dimensional alterations occur in the first six to eight weeks (Chen et al., 2004).

Placement of implants in fresh extractions sockets have failed to preserve alveolar ridge as shown by studies on dogs and humans (Araujo et al., 2005, 2006a, b; Botticelli et al., 2004). However, when implant placements are planned outside this window, then alveolar ridge preservation approaches help in maintaining the volume of ridge (Darby et al., 2008).

Regeneration of alveolar bone takes place via a sequential set of events:

- Formation of blood clot that contributes to haemostasis
- Inflammatory phase that is marked by recruitment of neutrophils, monocytes and macrophages
- Recruitment of cellular components that contribute to formation of new capillaries that marks the angiogenic phase

- Formation of new provisional matrix via newly recruited mesenchymal cells

- Remineralisation of provisional matrix that involves formation of new bone via osteoblasts. This process is regulated by different growth and differentiation factors leading to formation of woven and lamellar bone

- The last phase of remodelling is a continuous process. It is marked by bone coupling, where bone resorption is carried out by osteoclasts and osteoblasts deposit bone throughout life (Giannobile et al., 2019).

The following subsections describe the different bone and bone-substitute grafts and membranes that are used for ridge preservation.

1.6.1 Bone Grafts

Bone grafts can be described as biological or synthetic bone that is transplanted into an organism. These grafts can either be osteogenic, osteoinductive or osteoconductive.

1.6.1.1 Autologous Grafts

Autologous bone grafts are the patient's own bone harvested from a secondary site(s) such as the ramus or mental region (chin) of the mandible. This type of bone graft is considered as ideal (de Grado et al., 2018; Giannoudis et al., 2011). Autologous grafts are osteogenic, as they are thought to contain the patient's own cells, growth factors and biomolecules. They are also biologically safe, and biocompatible with corresponding mechanical and scaffolding characteristics. Collectively, autologous grafts possess osteogenic, osteoconductive and osteoinductive properties (Amini et al., 2012). Some limitations of these grafts include patient morbidity associated with a secondary surgical site and unpredictable rate of bone resorption (Haugen et al., 2019).

1.6.1.2 Allografts

Allografts are sourced from individuals (living or cadavers) of the same species but different genetic makeup. These grafts undergo decalcification, deproteinisation, irradiation and/ or freeze-drying and also sterilisation. These processes devitalise the

graft material removing cells and this reduces the osteoinductive characteristics. Some associated risks of allografts include infection transmission, immune reactions and high long-term rates of failure (de Grado et al., 2018; Winkler et al., 2018).

Allografts can be used as cortical wedges, chips or granules and cancellous bone powders. These grafts can be prepared as frozen, freeze-dried, mineralised and demineralised bone. Examples of allografts include mineralised bone allograft (Puros®), mineralised/ demineralised bone allograft (Raptos®), demineralised bone matrix (Grafton®, DBX®), freeze-dried bone allograft (FDBA) and demineralised freeze-dried bone allograft (DFDBA) (MFT®).

1.6.1.2.1 Fresh-frozen Bone Allografts (FFB)

These are cancellous bone that are frozen fresh and have the highest osteoconduction and osteoinduction (Dias et al., 2016; Macedo et al., 2012), however, they do carry risks of disease transmission.

1.6.1.3 Xenografts

Xenografts are derived from different species. Some biological drawbacks of xenografts include risk of disease transmission, host immune reactions (Schroeder & Mosheiff, 2011), and decreased osteoinductive capacity due to lack of viable cells attributed to manufacturing procedures (Zimmermann & Moghaddam, 2011). The most common xenografts used in dentistry are bovine in origin with no reports of risk of Transmissible Spongiform Encephalopathies (TSE) and Bovine Spongiform Encephalopathy (BSE) (Kim et al., 2013). A retrospective study over 20-years, involving more than 10,000 implants in more than 3,000 patients, has assessed implant therapy with and without ridge augmentation. The study reported that deproteinised bovine bone mineral was the most common bone graft used (>50%) and that DBBM grafts performed similarly to autologous grafts in terms of implant survival in a private practice setting (Knöfler et al., 2016). In a systematic review of 40 randomised controlled trials that assessed that the effect of different grafting materials on ridge resorption (Majzoub et al., 2019), the authors reported that similar amounts of resorption occurred with xenografts (1.47 ± 0.92mm) and allografts (1.52 ± 1.29mm). Xenografts have slow resorption rates and

thus preserve augmented bone; however, resorption rates can vary (Haugen et al., 2019). In a systematic review and meta-analysis, De Risi and colleagues (2015) reported that at seven months, xenografts showed comparable results in terms of residual graft materials (37.14%) to allografts (37.23%) (De Risi et al., 2015).

Some examples of xenografts include, anorganic porcine bone mineral (Gen-Os®), deproteinised bovine bone mineral (Bio-Oss®), anorganic bovine bone mineral (Osteograf/ N®), corraline calcium carbonate (Biocoral®) and porous hydroxyapatite (Interpore 200®). In this review of literature, the focus will be on Bio-Oss®.

1.6.1.3.1 Bio-Oss®

Bio-Oss® bone substitutes are made from the mineral component of bones from Australian cattle (bovine bone). The manufacturing process separates the organic components leaving the hard bony parts consisting of calcium compounds. The hard inorganic components are crushed into granules and treated with alkalis and chemicals under temperatures of up to 300^0C to remove any remaining organic components.

There are three main product formulations: Bio-Oss®, Bio-Oss Pen® and Bio-Oss Collagen®. Bio-Oss® and Bio-Oss Pen® are both available as small granules (0.25-1 mm) and large granules (1-2 mm). Bio-Oss Collagen® is composed of Bio-Oss® granules together with 10% porcine collagen and is available in three different sizes: 0.2-0.3 cm^3, 0.4-0.5 cm^3 and 0.9-1.3 cm^3.

Bio-Oss® is a commonly used xenograft for ridge preservation. Araujo and Lindhe (2009), in a dog study, demonstrated that unfilled sockets underwent three-fold more horizontal bone loss in comparison to sockets filled with Bio-Oss® (Araujo & Lindhe, 2009b). In another dog model, (Araujo et al., 2010) showed that when fresh sockets are filled with Bio-Oss® bone mineral is deposited in the collagen bundle of the provisional matrix. The study also reported osseointegration of Bio-Oss®, during the later phases of healing.

A 9-month observation of the effects of Bio-Oss® on healing of extraction sockets in humans, demonstrated the osteoconductive properties through bony ingrowth and

osseointegration with newly formed bone (Artzi et al., 2001). Additionally, the study reported the presence of osteoblasts inside the osteoid layer on the borders of the newly formed bone and graft material.

Overall, Bio-Oss® is a widely used and well documented xenograft for ridge preservation and augmentation,

1.6.1.4 Natural Biomaterials

Natural polymers are used as bone grafting materials as they are analogous with the host extracellular matrix with regard to chemical make-up and offer high osteoinduction (Haugen et al., 2019). Natural polymers can be classified into three types; proteins (collagen, gelatine, fibrinogen, elastin); polysaccharides (glycosaminoglycans, cellulose, amylose) and polynucleotides (DNA, RNA).

These bone grafts possess high biocompatibility with reduced risk for host immunoreactivity. Studies have demonstrated that natural polymers have the potential for differentiating mesenchymal stem cells into osteoblasts (Chung & Burdick, 2009; Wang et al., 2005). There are additional concerns regarding the mechanical characteristics of the newly formed bone (Hannink & Arts, 2011; Mano et al., 2007).

1.6.1.5 Synthetic Materials

Synthetic materials can be either polymers or bioceramics. Synthetic polymers possess biomechanical and biodegradability characteristics that can be controlled and adjusted. The porosity, chemical and physical properties and immunoreactivity can be controlled better than other bone graft substitutes (Fuchs et al., 2001; Kretlow & Mikos, 2007). Aliphatic polyesters such as poly(lactic acid) (PLA), poly(ε-caprolactone) and poly(glycolic acid) copolymers and their derivatives are the among the most researched synthetic polymers. Osteoconductivity, resorption time and changes in pH are among some of the concerns of these grafting materials (Haugen et al., 2019).

Some examples of synthetic bone substitutes include, bioactive glass (PerioGlas®), beta-tricalcium phosphate (Vitoss®) and dicalcium phosphate dehydrate (Eurobone®).

1.6.2 Dental Membranes

Barrier dental membranes were first used in 1988 with the idea that different cells proliferate at different rates and hence, it is important to exclude particular cells that proliferated at a faster rate. This exclusion allowed cells from all tissues compartments of the periodontium to proliferate, especially bone forming cells that have a slower rate of proliferation (Dahlin et al., 1988; Melcher, 1976). Barrier membranes can broadly be classified as non-resorbable or resorbable. The key features of barrier membranes in relation to periodontal wound healing are based on their ability to stabilise the blood clot, maintain space and allow primary intention healing (Susin et al., 2015).

1.6.2.1 Non-resorbable Membranes

Non-resorbable membranes allow clinicians to control the retrieval time of the membrane. However, membrane retrieval requires a second stage surgery that adds to patient morbidity and potential damage to regenerated tissues (Tatakis et al., 1999). The most common complication involves membrane exposure (Murphy, 1995). There are two main types of non-resorbable barrier membranes, titanium and polytetrafluoroethylene (PTFE).

Titanium barrier membranes are physically strong, rigid and yet malleable. These characteristics help in maintaining space, preventing collapse and adaptation over defects. Titanium mesh has shown predictable space maintenance in different studies (Becker et al., 1996; Zitzmann et al., 1997).

PTFE is a fluorocarbon polymer that is non-porous, inert and biocompatible (Sculean et al., 2008). There are two main types of PTFE, expanded-PTFE (e-PTFE) and titanium-reinforced high-density PTFE (Ti-d-PTFE). The e-PTFE membranes have been shown to be biologically stable and effective in periodontal regeneration (Hammerle & Jung, 2003; Piattelli et al., 1996). Ti-d-PTFE has small pore size which reduces bacterial ingress in case of membrane exposure (Ronda et al., 2014).

Clinical, histological (Arbab et al., 2016) and biomolecular (Mandarino et al., 2018) studies have shown that non-resorbable and resorbable barrier membranes have

comparable healing when used for ARP. However, due to the morbidities associated with non-resorbable membranes, they are not routinely used for ridge preservation.

1.6.2.2 Resorbable Membranes

The safety and effectiveness of resorbable membrane are dependent on the degradation end-products. As the membranes resorb their end-products can trigger immunological reactions that may consequently cause adverse effects such as bone resorption (Younger & Chapman, 1989). Resorbable membranes can be further classified into synthetic and natural types.

Poly-α-hydroxy acids are the most common material for synthetic resorbable membranes. Some advantages of synthetic resorbable membranes are tuneable biodegradability, easy manageability and drug encapsulation (Sanz et al., 2019). However, there are possibilities of immunological reactions that can cause resorption of regenerated bone. Resorption rates are dependent on the type of polymer used. Some examples of synthetic resorbable membranes include Epi-Guide®, Resolut LT®, Artrisorb®, Guidor®, vicryl periodontal mesh® and Mempol®.

Natural resorbable membranes are made up of human or animal collagen. They can either be non-cross linked or cross-linked. When non-cross-linked natural collagen membrane degrade they have not been shown to have adverse effects on tissues. However, they do lack physical strength that can affect their space maintenance capacities and can resorb quite rapidly (Sanz et al., 2019). Collagen membranes undergo various physical/ chemical cross-linking processes to decrease the resorption rates and enhance mechanical properties. However, the consequence of chemical cross-linking is inflammation associated with chemical remnants such as amides and aldehydes. The origin of the collagen material and the processing protocols such as decellularisation, sterilisation and cross-linking methods, can all contribute to the predictability of the collagen membranes (Sanz et al., 2019).

Chitosans are polysaccharides that are made up of glucosamine and N-acetylglucosamine (Kweon, et al., 2003). Chitosans barrier membranes are non-woven and porous with good handling properties. These membranes have shown similar

regenerative capacities similar to collagen membranes (Sanz et al., 2019), and hold promise.

1.6.2.2.1 Bio-Gide®

Bio-Gide barrier membranes are double layered and composed of type I and III collagen from porcine skin. The resorption time for these membranes is about eight weeks (Schliephake et al., 1994). They come in three sizes: 13 x 25 mm, 25 x 25 mm and 40 x 50 mm. Various clinical studies have demonstrated that when used in combination with bone graft, Bio-Gide® collagen membranes have positive clinical outcomes for alveolar ridge preservation (Lei et al., 2015; Zhan et al., 2017; Zhao et al., 2018; Zhao et al., 2019).

1.7 Animal Models

Animal models are a useful tool for assessing periodontal disease processes and to evaluate the effects of various treatment modalities. Healing of periodontal and peri-implant tissues and regeneration after periodontal surgery using different biologic materials can be studied in animals. These can provide useful insights for possible tissue engineering strategies involving different periodontal tissue compartments such as the epithelium, connective tissues and alveolar bone (Bosshardt & Sculean, 2009). The biology of periodontal and peri-implant healing is complex hence animal models are essential to successfully interpret use of regenerative materials and dental implants before clinical application.

Many animal species such as mice, rabbits, hamsters, dogs, pigs, sheep and non-human primates have been used as models in periodontal research. There are many factors that need to be considered when selecting a particular species for research. A well-defined research question is necessary before selecting a model to answer those questions. Schimandle and Boden (1994) have outlined factors such as cost of acquisition, animal care, availability, appropriateness to society, acceptance of captivity and animal housing need to be considered (Schimandle & Boden, 1994). The study conditions should be in alignment with the Animal Protection Acts of the governing bodies; this should include housing facilities, dietary requirements and lighting. Appropriate care of animals by trained personnel before, during and after the study should be taken into consideration. In terms of general health of the animals, overall ease of maintenance and handling, resistance to infections, inter-animal interactions and ability to undergo surgery needs to be considered (Pearce et al., 2007).

For studies that investigate bone regeneration the species chosen should have similar bone characteristics in terms of bone microstructure and composition, bone modelling and remodelling. This is especially important when inferring results from animal studies to humans. The size of the animal model chosen is equally important to determine the number and size of implants. Hazzard and colleagues (1992) highlighted that for any field of research there is no gold standard in terms of animal models, and no animal model is suitable for all for purposes (Hazzard et al., 1992).

Pearce and colleague (2007) consolidated the characteristics of bone from different animals and their similarities to human bone.

Table 1: Key Attributes in Terms of Similarities between Animal and Human Bone

	Rabbit	Dog	Pig	Goat	Sheep
Macrostructure	+	++	++	+++	+++
Microstructure	+	++	++	+	+
Bone Composition	++	+++	+++	++	++
Bone Remodelling	+	++	+++	++	++

+ least similar, ++ moderately similar, +++ most similar
Adapted from Pearce, et al., 2007

1.7.1 Small Animals

Despite having limited similarities to human bone and dentition, small animals have been used in dental research. These animals can be studied *in vivo* using genetically produced transgenic and knockout animals (Graves et al., 2008) and are also cheaper compared to larger animals (Kantarci et al., 2015). Small animals such as rodents and rabbits have been used in periodontal regenerative studies.

1.7.1.1 Rodents

Rodents include mice, rats and hamsters. Their dentition is comprised of one incisor and three molar teeth in each quadrant. Mice are the smallest animal model used in dental research, and are very cost-effective, especially when compared with large animals. The mouse calvaria model has been used in different periodontal research including regenerative studies (Graves et al., 2008). Similar studies have been carried out to evaluate soft and hard tissue healing and assess the role of various molecules on bone turnover and regeneration in rats. A number of studies have been carried out on alveolar ridge preservation in rats using bone grafting materials (Alikhani et al., 2016; de Lima et al., 2000; Hahn et al., 1988; Mayer et al., 2018; Mott et al., 2002; Pinholt et al., 1990, 1991).

For immunohistochemical studies, antibodies against mouse are readily available. This allows various immunohistochemical end-points to be studied in mice, including knockout studies against different genetic backgrounds. The availability of the mouse genome also allows the roles of genes in disease regulation, inflammation and regeneration including periodontal disease to be studied.

1.7.1.2 Rabbits

Rabbit models have been used to study the effects of inflammation, periodontal wound healing and regeneration. The main disadvantage of this model is the size of the animal for evaluating multiple implant materials (Pearce et al., 2007). There is limited literature that demonstrates using alveolar bone in rabbits for periodontal regeneration or dental implants (Abrahamsson, 2011; Caiazza et al., 1991; Slotte et al., 2003). Some studies have reported that rabbit models are not adequate for periodontal ligament regeneration (Oortgiesen et al., 2010; Tyrrell et al., 2002).

1.7.2 Large Animals

Large animals have similar dental anatomy to that of human, and this allows better generalisation and application to clinical settings. Animals such as dogs, nonhuman primates, miniature pigs and sheep and have used in periodontal research.

1.7.2.1 Dogs

Dogs are a well-studied model for periodontal research because the periodontal tissues and size of teeth in dogs are similar to humans (Kantarci et al., 2015). In the permanent dentition dogs have three incisors, one canine, four premolars and three molars in the lower arch and two molars in the upper arch (Navia, 1977). Dog models are widely used in wound healing, dental implants and regenerative studies (Wikesjo et al., 1994). The use of dog models for guided tissue regeneration (GTR) studies dates to the 1980s (Nyman et al., 1980). A number of studies have shown the evaluation of bone substitutes (Levy et al., 1981; Plotzke et al., 1993; Sugaya et al., 1990; Sugaya et al., 1989) and the efficacy and biocompatibility of various membranes (Araujo & Lindhe, 2005, 2009a, 2011; Cardaropoli et al., 2003; Fleisher et al., 1988; Haney et al., 1993; Magnusson et al.,

1988, 1990; Nalbandian & Hellden, 1982; Pitaru et al., 1988; Tal et al., 1991; Tal & Pitaru, 1992; Tan et al., 2019) using this model.

The use of this model has decreased over the years due to ethical concerns around the use of companion animals; this model has however, been very useful for studying tissue responses to regenerative periodontal treatment.

1.7.2.2 Goats

Goat models are usually used for orthopaedic research and are easier to acquire as they are considered consumption animals. Leung and colleagues (2001) reported that goats are able to tolerate certain environmental conditions like hot and humid conditions better than other animals like sheep (Leung et al., 2001). Studies have described goats to be a suitable model for assessing implants and materials as they have similar metabolic and bone remodelling rates as humans (Anderson et al., 1999; Dai et al., 2005; Spaargaren, 1994). This model, therefore, can also be suitable for assessing periodontal regeneration.

1.7.2.3 Miniature Pigs

These animals are similar to humans both anatomically and physiologically and also economically offer advantages over other large animals. Miniature pigs have been used for periodontal and implant studies (Lynch et al., 1991; Schliephake & Neukam, 1991; Schliephake et al., 1991a,b). Testing of different bone substitutes and membranes have been studied using this model (Buser et al., 1998; Hamp et al., 1972; Naaman Bou-Abboud et al., 1994; Schliephake & Aleyt, 1998), and the healing process has been reported similar to humans (Buser et al., 1998).

1.7.2.4 Nonhuman Primates

Nonhuman primates have oral structures that are similar to that of the human, with similar permanent dentition, however, the size of the teeth are smaller. The dentogingival and periodontal fibres have similar organisation to humans. These models have been described as suitable for periodontal regenerative procedures (Caton et al., 1994; Schou et al., 1993). Min and colleagues (2016) used a monkey model for an

alveolar ridge preservation study and reported similar findings to human and dog studies (Min et al., 2016). Similar studies have been carried on monkeys to assess periodontal healing using biomaterials (Drury & Yukna, 1991; Karatzas et al., 1999; Su et al., 2017), and guided tissue regeneration (Kostopoulos & Karring, 2004).

Even though nonhuman primates provide a satisfactory model for studying different aspects of periodontal diseases and treatment modalities this model has some major drawbacks. It is expensive to buy and maintain monkeys and at the same time they can be violent and can carry diseases, especially those that are captured wild (Schou et al., 1993). Monkeys are also susceptible to systemic infections and diseases which make post-operative healing and care difficult (Weinberg & Bral, 1999).

1.7.2.5 Sheep

Sheep have a permanent dentition that consists of three pairs of incisors and one pair of canines on the mandibular arch and three premolars and three molars on each quadrant. The use of sheep in orthopaedic surgery has increased over the years (Pearce et al., 2007). Sheep are a suitable model for periodontal and implant research because their weights are similar to humans and long bones in sheep provide appropriate dimensions for implants and prosthesis (Newman et al., 1995).

Histologically, sheep bone contains osteons that are smaller than 100 μm in diameter with about two central blood vessels and no cement line (deKleer, 2006). This is different from human bones that mostly contain secondary bone (Eitel et al., 1981). Sheep bone has also been reported to be denser than that of humans (Nafei et al., 2000). Ovine age plays an important role determining the amount of bone remodelling. The mechanical and physical characteristics of immature sheep are similar to humans (Nafei et al., 2000). It is therefore important to include sheep with similar age within a study as age dissimilarities can make comparisons difficult.

There have been a number of studies using the sheep model for evaluating the effects of different biomaterials on alveolar ridge preservations procedures (Adeyemo et al., 2008; Paknejad et al., 2008; Rodriguez y Baena et al., 1998; Schmitt et al., 2008).

A sheep model for implant research has been validated (Duncan, 2005) and has been utilised in various periodontal healing studies (Baharuddin et al., 2015; Liu et al., 2016). Liu and colleagues (2016) were the first to use the tooth extraction socket model in sheep and have reported that sheep models are suitable for assessing bone graft healing.

1.8 Hypothesis and Study Objectives

1.8.1 Aim

The aim of this investigation was to investigate the key osteogenic factors expressed during healing in a tooth socket model.

1.8.2 Hypothesis

Use of a bone xenograft and resorbable membrane will result in increased expression of key osteogenic markers in an extraction socket, compared to a non-grafted and non-covered socket.

1.8.3 Objectives

1. To identify the cells associated with the expression of the key markers of osteogenesis, RANK, RANKL and OPG in sockets grafted with bone xenograft (Bio-Oss®) and resorbable membrane (Bio-Gide®) or in untreated tooth sockets, at 4-, 8- and 16-weeks post-surgery.

2. To determine mRNA expression levels for RANK, RANKL, OPG and regulators of osteoblast differentiation Col1A1, TIMP3, Sp7 and Msx2 in the presence of Bio-Oss® and Bio-Gide® compared with untreated tooth sockets at 4-, 8- and 16- weeks post-surgery.

Chapter 2 Materials and Methods

The materials and methodology used to compare the healing of tooth socket grafted with Bio-Oss® and natural bone healing in a sheep model are outlined in this chapter.

2.1 Ethical Considerations

All aspects of the study were approved by the Otago University Animal Ethics Committee (Application No. 75/2017) in accordance with the New Zealand Regulations (New Zealand Animal Welfare Act 1999). All investigators undertook the Module 1 - Ethics and Legislation AWO101 course prior to the commencement of the study.

2.2 Surgical Materials and Equipment

2.2.1 Bio-Oss®

Bio-Oss® (Geistlich, Switzerland) is a porous bone mineral matrix that is produced by removing the organic components from bovine bone. A spongiosa (cancellous) granular type Bio-Oss® with particle sizes ranging from 0.25-1.00 mm was used (Figure 2).

Figure 2: Geistlich Bio-Oss®.

2.2.2 Bio-Gide®

The Bio-Gide® (Geistlich, Switzerland) membrane was cut to approximate size of the socket (Figure 3). The corners of the membrane were rounded for easy manipulation.

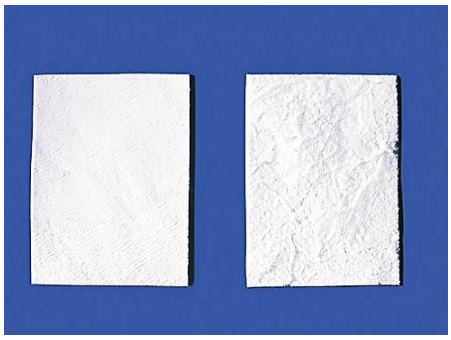


Figure 3: Bio-Gide® Membrane
The bi-layer structure, (Left) dense side and (Right) porous side.

2.2.3 Implant Bur

Implant sockets were created by extracting teeth. The size of the sockets were standardised using an implant bur (catalogue number D-6.0 TP-11 Southern Max Ex-Hex system, Southern Implants, Irene, South Africa) to a depth of 11 mm x 6.0 mm wide at the coronal portion.

2.2.4 Trephine

Tissue samples for RNA analysis were recovered using a 2 mm diameter trephine (Meisigner, Neuss, Germany) from the third premolar on the right side of the mandible.

2.3 Study Design

A experimental study was carried using a sheep (*Ovis aries*) model. Natural bone healing was compared to healing of the tooth extraction socket in the presence of Bio-Oss® at 4, 8 and 16 weeks. The study design is outlined in Figure 4.

2.3.1 Selection Criteria

The study group included 30 mature (three to four years of age) female, full-mouth and barren sheep. Sheep which were underweight <75 kilograms or with lamb at foot, had foot rot, or had broken-mouth periodontitis (as shown by loss of one or more incisors) were excluded from the study. To reduce outbred heterogeneity, all animals were selected from one farm and at random from a larger pool at the farm site (AgResearch, Invermay).

2.3.2 Randomisation

Each animal was assigned a unique three-digit numeric code that was identifiable from the tags placed on the animals' ear. Randomisation of the treatment was conducted using a Latin Square design as per Appendix I. Treatment allocations for each animal were verified at the time of surgery.

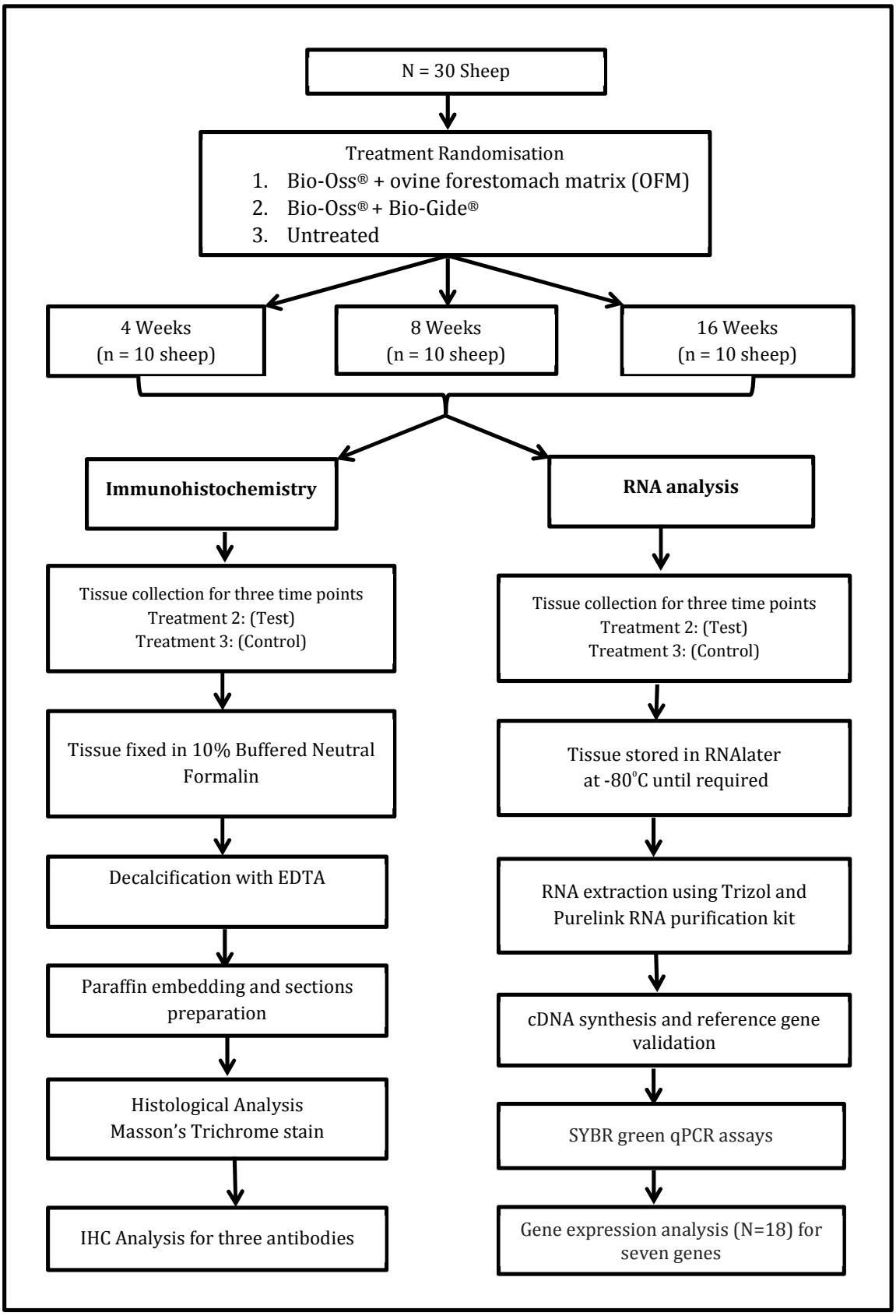


Figure 4: Study Flow Diagram.
Two main aspects of the study will involve immunohistochemistry and gene expression analysis.

2.4 Treatment Allocation

The first, second and third mandibular premolars were selected as the experiment sites, as the roots of these teeth resemble those of the human teeth and surgical accessibility is restricted past the third premolar without a cheek incision. The surgical sites are shown below (Figure 5).

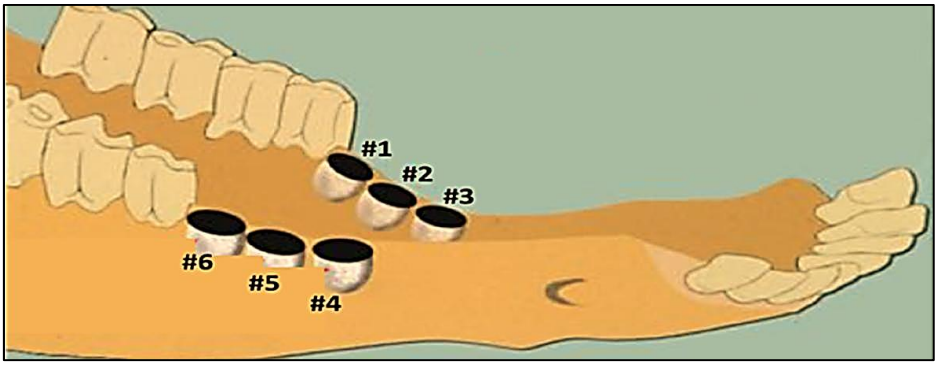


Figure 5: Premolar Surgical Sites.
(#**1)** Left third premolar (LP3). (#**2**) Left second premolar (LP2). (#**3)** Left first premolar (LP1). (#**4)** Right first premolar (RP1) (#**5)** Right second premolar (RP2). (#**6)** Right third premolar (RP3).

Three premolars were extracted bilaterally and a total of six defects in each animal were randomized and allocated the following treatments (Figure 6):

Treatment A - Geistlich Bio-Oss® (particle size: 0.25 to 1.00 mm) + Ovine Forestomach Matrix (OFM)

Treatment B - Geistlich Bio-Oss® (Particle size: 0.25 to 1.00 mm) + Geistlich Bio-Gide® Membrane

Treatment C - Untreated

Only the sockets from the right side of the mouth that were treated with Bio-Oss® and Bio-Gide® (test) or left empty for natural healing (control) were analysed in this study. The sites treated with an experimental membrane (OFM) were analysed as part of a separate study.

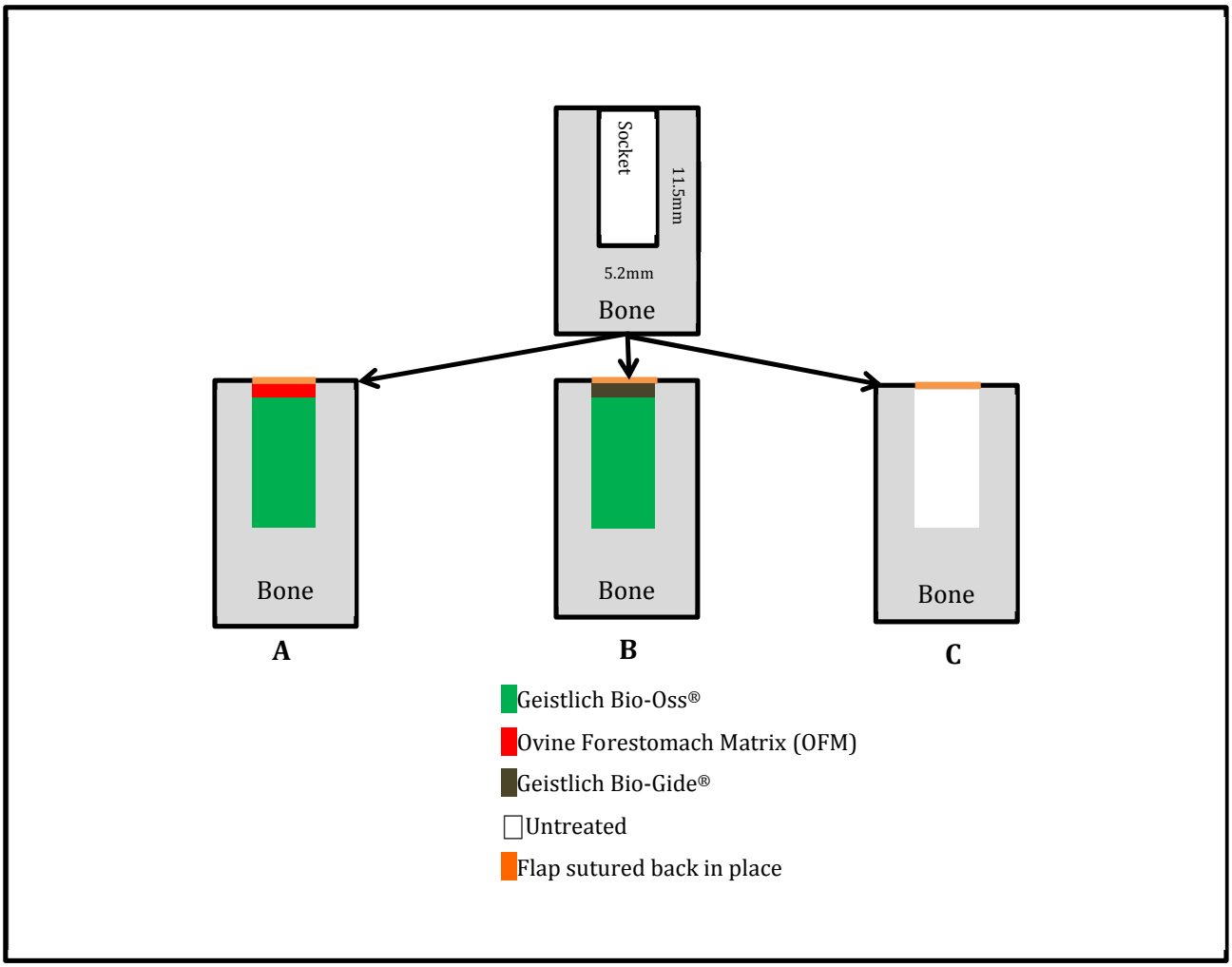


Figure 6: Schematic presentation of the treatment allocation

2.5 Surgical Protocol

2.5.1 Pre-Surgical Work-up

The sheep were treated with anthelmintic and clostridia vaccines, prior to transportation to the research facility. For acclimatisation, the sheep were kept in approved indoor housing at the research facility for a minimum of seven days prior to surgery and provided with hay, pellets and water. Food was withheld 12 hours before surgery. Surgery was performed under standard sterile operating techniques at Invermay Agricultural Research Centre, Mosgiel.

All sheep received a subcutaneous dose of the antibiotic Trimethoprim (Amphoprim injection 1 mL/15 kg, Virbac New Zealand Ltd., Auckland, New Zealand) 1 hr before surgery.

2.5.2 General Anaesthesia

General anaesthesia was induced via the cephalic vein with Diazepam (0.2 mg/kg) and Ketamine (2 mg/kg) which was maintained with Isoflurane (2.5 to 3.5% to effect). Sheep were positioned on a mobile, adjustable table and a sandbag was used for head support. The animals were intubated by a veterinarian and the rumen was compressed with a stomach tube and the contents allowed to drain into a bucket on the floor. Animals were lifted and rotated by veterinarians on the platform to allow access to the alternate surgical site during surgery. The animals were monitored by a veterinarian throughout the surgical procedure.

2.5.3 Disinfecting Surgical Site

The face was trimmed of wool and prepared with Betadine. The oral cavity was prepared with sterile gauze soaked with 0.2% chlorhexidine gluconate solution (Savacol®; Colgate-Palmolive, NZ) for disinfection.

2.5.4 Local Anaesthesia

A local dental anaesthetic infiltration (Lignospan® Lignocaine Hydrochloride B.P. 2% with Adrenaline 1:80,000) was administered adjacent to the operative sites for the purpose of decreasing immediate post-operative discomfort and providing a degree of haemostasis during the surgery. Additional local anaesthesia was also administered following the completion of the surgical procedure.

2.5.5 Tooth Extraction Protocol

A minimally traumatic approach was adopted for the extraction of the mandibular premolars. A full thickness mucoperiosteal flap was raised buccally and lingually from the first mandibular molar extending anteriorly to approximately 1 cm mesial to the first mandibular premolar. Access to the contact area between the teeth was gained by means of dental elevators gently tapped with a surgical mallet. Molar extraction forceps together with Coupland's and Cryer's elevators and luxators were used to mesially luxate and extract the premolars. Special care was taken not to damage the cortical plates, inter-radicular bone and to avoid root fractures. Complete tooth removal was verified radiographically with periapical radiographs post-extraction. Any remaining fractured roots were removed using root pick elevators and the sockets were curetted and then re-imaged. A Rinn® holder paralleling technique and dental intraoral x-ray film (Kodak; Size two) were used for all imaging.

2.5.6 Preparation of Grafting Site

As the size of each socket is different and also varies across individual animals, the sockets were standardised using implant burs to at least 11 millimetres deep x 5.2 millimetres wide at their coronal portion (D-6.0 TP-11 Southern Max Ex-Hex system, Southern Implants, Irene, South Africa). The width of the ridge at the crestal level was measured just mesially to each defect using a Williams' periodontal probe (Figure 7).

2.5.7 Grafting of Extraction Sites

Bio-Oss® was introduced in the prepared socket using a 2cc syringe that was modified by cutting off the adaptor. The modified syringe was used to draw blood from the extraction socket, which was then mixed with Bio-Oss® that had been moistened with sterile water (1-2 mL). Each defect was packed with approximately 0.5 grams of Bio-Oss®. The Bio-Gide® collagen membrane was trimmed to size per standard practice and placed over the grafted defect. The buccal and lingual mucoperiosteal flaps were sutured closed using resorbable sutures (Vicryl 3-0, Ethicon Inc., Somerville, MA, USA). Untreated control defects received no bone grafting material or collagen membrane and were closed with sutured flaps only. Intraoral radiographs were taken immediately post-operative.

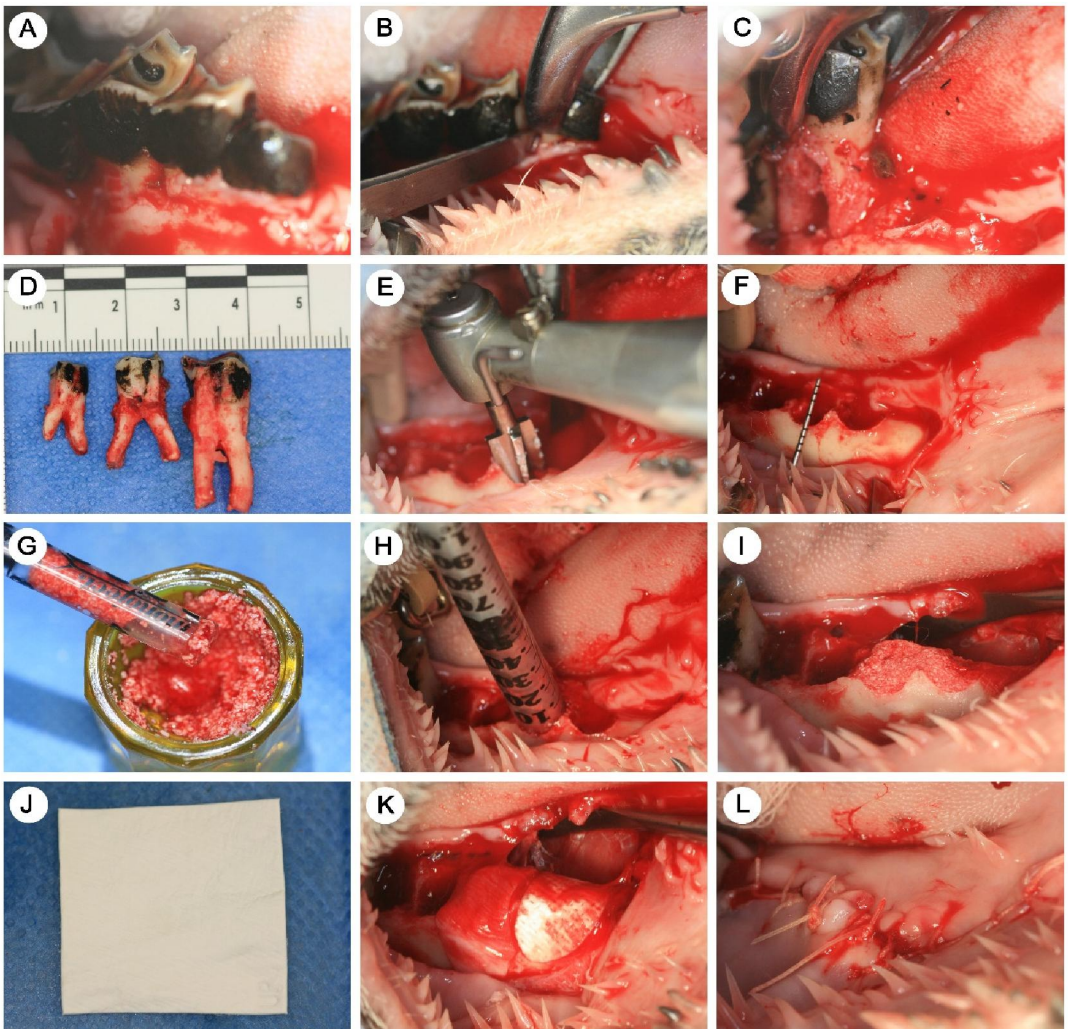


Figure 7: Surgical Protocol.
(A) Surgical site identified. (B) Tooth elevated. (C) Forceps used to extract the teeth. (D) First, second and third premolars post-extraction. (E) Implant burs used to prepare the socket to ≥11 millimetres deep x 5.2 millimetres wide at their coronal portion. (F) William's periodontal probe used to measure width and depth of the socket. (G) Prepared Bio-Oss® mixed with blood drawn from the socket. (H) Modified syringe used to pack Bio-Oss® into the socket up to the alveolar crest (I) Socket packed with Bio-Oss®. (J) Prepared Bio-Gide® membrane. (K) Membrane in place. (L) Flap sutured back over the socket.

2.5.8 Post-Surgery Care and Recovery

Post-surgery, animals were housed in sheep pens for five to seven days and given food (small pellets or muesli-type feed) and water twice daily. The anti-inflammatory Carprofen (4 mg/kg; subcutaneous injection) for pain relief, and the antibiotic Amphoprim (Trimethoprim sulpha; 1 mL/15 kg subcutaneous), were given once daily

for three days. An antiseptic solution (Chlorhexidine gluconate (10 cc 0.2% aq) was also applied daily to the intraoral surgical sites via a 20 mL syringe. The veterinary staff monitored and documented any anomalies for a minimum of three days post-surgery. Monitoring included food and water intake, behaviour and any complications to the defect site or animal health. Health and behaviour were assessed by observing animals' mannerism including, but not limited to, gait and movement, posture, respiration, vocalisation and faecal consistency. Defect complications were assessed by observing site haemorrhage, oedema and infection. Central nervous system (CNS) complications, indicating an anaesthetic complication immediately after surgery, or other behavioural indicators, such as self-mutilation were closely monitored in the first 48 hours after surgery and with any complications noted.

2.5.9 Post-Operative Recovery to Euthanasia

Following post-operative recovery, up until euthanasia, the animals were housed in farm paddocks. The animals' welfare was monitored daily by a qualified veterinarian or a veterinarian nurse.

2.5.10 Euthanasia

Following healing periods of 4-, 8- and 16-weeks, 10 animals per time point were euthanized. General anaesthesia was induced with an intravenous infusion of Diazepam (0.2 mg/kg) and Ketamine (2 mg/kg, intubated orally and general anaesthesia was maintained using isoflurane 2.5 to 3.5% to effect.

Prior to catheterisation and fixation, tissues samples for analysis of RNA expression were trephinated from the third premolar site, as discussed in detail below (Section 2.8).

The animal was then placed on a trolley-table in a supine position, with the neck slightly overextended. The exposed neck region was closely shaven with a trimmer. A trans-cutaneous incision, approximately 10 cm in length was made; a blunt dissection of the underlying strap muscles of the neck to the level of the carotid artery was then made. The carotid arteries were then cannulated bi-laterally, using 14G x 2" gauge indwelling

catheters (Optiva™, Smiths Medical, UK). The catheters were ligated firmly, while maintaining patency, to prevent dislodging the cannula.

An anaesthetic overdose (isoflurane) was used to euthanize the animals. The jugular veins were severed, and the carotid arteries were perfused with sterile saline (2 L; Baxter Healthcare Pty Ltd, Australia) containing 1.5 mL of 5000 iu heparin. This was followed by 10% neutral buffered formalin (NBF; BioLab Ltd., Auckland, New Zealand; 2 L; 0.4%) perfused bilaterally to fix the tissues. Following the anaesthetic overdose, exsanguination and fixation the euthanasia time was recorded and the surgical sites were identified.

2.5.11 Tissue Harvesting

Post-mortem the formalin-perfused tissue specimens were harvested and resected *en bloc*. The mandible was disarticulated and removed, and the overlying skin dissected off using a large scalpel. The mandible was divided at the mid-line with a saw and the two halves labelled; with only the right side used for this study.

2.6 Specimen Preparation for Immunohistochemistry

2.6.1 Tissue Collection

Tissue specimens for histology were collected from the right side of the mandible from all 30 sheep and were fixed *en bloc* in 10% neutral buffered formalin for 48 hrs at 4°C with gentle rotation. A total of 60 tissue samples were collected with 20 from each time point. After 48 hours, the tissue blocks were transferred to 20% ethanol. Radiographs taken using a Schonander® skull unit to allow localization of each socket. Each extraction socket was located on the radiograph and a reference mark made on the mandible. The individual sockets were then separated using a manual coping saw.

2.6.2 Decalcification

The decalcification process used in this study was described by Baharuddin and colleagues (2015). Each socket was placed in a labelled pottle containing 10% ethylenediamine tetra-acetic acid (EDTA) pH 7.4 (150 mL; Appendix II) and agitated at 4°C on a rotating platform. The EDTA solution was changed every 48 hrs. Decalcification was considered complete when no sign of radiopaque tissue was seen on radiographs and a negative oxalate test.

An oxalate test was employed to confirm decalcification. A sample of the EDTA decalcification buffer (5 mL) was taken from the pottle containing the bone and concentrated hydrochloric acid (HCl) (Merck®, Germany) added to the solution until a pH 3.2 - 3.6 was achieved. Litmus paper was used to determine the pH of the solution. A solution of 3% di-ammonium oxalate monohydrate (5 mL; Extra pure; Merck®, Germany) was then added and left to incubate at RT for 30 mins. A cloudy appearance indicated incomplete decalcification (see Appendix II for buffer recipe). The samples were radiographed every two weeks (or as required) to assess the extent of decalcification using the Gendex dental systems (Monza, Italy) on Kodak Intraoral dental films (Carestream Health, New York, USA). The films were exposed at 38 cm focal length for 0.16 seconds and processed in an automatic processing machine.

Following decalcification, the tissue was placed in a paraffin embedding cassette with a paper label. Correct orientation of the sample was ensured with a blue dye marking the cutting surface. The cassettes containing the tissue were then placed in phosphate buffered saline (PBS) buffer (1742570, Gibco® Invitrogen) and agitated for 24 hr at 4°C on a rotating platform.

2.6.3 Paraffin Embedding

The embedding process was carried out using an automatic processing machine (Excelsior™ ES tissue processor, Thermo Scientific™, Waltham, USA). The specimens were prepared using Routine Overnight program cycles with a series of formalin, graded ethanol, xylene, and paraplast wax (56°C melting point) steps. See appendix III for protocol. Following processing, the tissue was embedded in molten paraffin (62°C) in an embedding machine (Histostar™, Thermo Scientific™). The resulting paraffin blocks orientated the socket in the buccal-lingual direction along the long axis of the tooth.

2.6.4 Sectioning

A Leica RM 2025 microtome (Leica Microsystems Inc. Deerfield, USA) with disposable feather microtome blades (#S35, 17120676), was used to cut 4 µm thick sections from the centre of the prepared blocks. Using tweezers, the cut wax ribbons were floated on 30% ethanol for 20 secs followed by a 50-55°C waterbath for 60 secs to assist floating the sections onto SuperFrost Ultra Plus™ Adhesion Slides (J3800AMNZ, Thermo Scientific™). A number of slide adhesion chemistries were tested to ensure optimal tissue adherence (Appendix III). The slides were labelled and incubated for 20 min at 45°C to dry the slides and then placed at 60°C overnight. The prepared slides were placed in an air-tight container and stored at 4°C until required.

2.6.5 Haematoxylin and Eosin (H & E) Staining

H & E staining was carried out on an automatic stainer (Shandon Varistain 24-3, United Kingdom) at the Pathology Department, Otago University. (See appendix III).

2.6.6 Trichrome Staining

Trichrome staining was carried using the Trichrome Staining Kit (ab150686, Abcam) at the Pathology Department, Otago University. (See appendix III).

2.6.7 Cover-slipping

Following staining the tissue sections were covered with a protective glass coverslip. Two drops of slide mounting medium (DPX, Mercek) was used to cover the section on the slide before placing a glass coverslip on top (HD LD2450 1.01PO, Grale HDS, Germany). All cover-slipping was carried out in a fume cupboard.

2.7 Immunohistochemistry

RANKL, RANK and OPG protein detection was carried out using immunohistochemistry. Due to the unavailability of sheep antibodies, human or rat antibodies were used. Antibodies were selected based on the sheep protein sequence homology with the human or rat sequence. Protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) and aligned using BLAST® software (www.ncbi.nlm.nih.gov). It was necessary to optimize antigen retrieval methods for each of the antibodies.

2.7.1 Trial Immunohistochemistry

Prior to running the final tests, several trials were run to determine appropriate working concentrations of the antibodies. Trials were also conducted to investigate which slides provided the best adherence of decalcified sheep alveolar socket tissues during immunohistochemistry. The following variables were tested (full protocol is given in Appendix III):

i. Different types of slides: Uber slides, Adhesive Trajan (806052N-SP3A, Trajan Scientific and Medical); Crest Adhesive (803158R-SP3A, Matsunami Glass Ltd); and Superfrost Ultra Plus® (J3800AMNZ, Thermo Scientific).

ii. Incubation of slides post paraffin embedding (incubated at 60^0C overnight or incubated at RT).

iii. Heat retrieval temperatures and time (decloaking chamber at 80°C for 15 minutes or water bath at 80°C for five minutes).

iv. Trypsin pre-treatment times were trialled at five and 15 minutes.

Superfrost Ultra Plus® (J3800AMNZ, Thermo Scientific) slides, that were incubated post tissue application overnight at 60°C, showed optimum tissue adherence. For the RANKL and RANK protocols, heat retrieval 80°C for 15 minutes in the Decloaking chamber provided optimum tissue adherence. A trypsin pre-treatment time of 10 minutes was optimal for OPG.

Prior to confirming the final primary antibody for RANKL and RANK, a different set of antibodies were trialled. For RANKL and RANK, goat anti-mouse (AF462 and AF692, R&D systems) at concentrations of 0.2 µg/ mL (1:1000) dilution and 2 µg/ mL (1:100) dilution were trialled. A set of matched negative controls, goat IgG (ab37373, Abcam) were also tested at the same concentrations as the primary antibodies. Two different secondary antibodies, polyclonal biotinylated rabbit anti-goat IgG H&L (ab6740, Abcam) 2 mg/mL used at a dilution of 1:1000, and polyclonal biotinylated rabbit anti-goat IgG F(ab')2 (31753, Invitrogen), 1.4 mg/mL at dilutions of 1:500, 1:1000 and 1:5000 were trialled. However, these set of antibodies were not successful and hence were not used in the final experiments.

The OPG antibody concentrations was trialled at 2.5 µg/mL (dilution 1:400) and 5 µg/mL (dilution 1:200). The negative controls were also tested at the same concentrations, mouse IgG 2.5 µg/mL (dilution 1:160) and 5 µg/mL (dilution 1:80).

2.7.2 Primary antibody Selection

2.7.2.1 RANKL Antibody

A polyclonal rabbit anti-mouse RANKL antibody (ab216484, Abcam) was used in the final experiments. Several antibody concentrations were tested (2.5 and 5 µg/mL) with a final RANKL antibody concentration of 5 µg/mL (1:200 dilution) selected for the study samples.

2.7.2.2 RANK Antibody

A polyclonal rabbit anti-human RANK antibody (PA5-80145 CD265, Invitrogen) was used in the final experiments. Several antibody concentrations were tested (2.5 and 5 µg/mL) with a final RANK antibody concentration of 5 µg/mL (1:100 dilution) used with the study samples.

2.7.2.3 OPG Antibody

A monoclonal mouse anti-human OPG antibody (NB100-56505, Novusbio) was used. This antibody had been previously validated on sheep (Baharuddin et al., 2015).

Antibody concentrations of 2.5 and 5 µg/mL were tested, with a final OPG concentration of 5 µg/mL (1:200 dilution) used with the study samples

2.7.3 Secondary Antibody Selection

The secondary antibody for both RANKL and RANK was a polyclonal biotinylated goat anti-rabbit IgG H&L (ab6012, Abcam), conjugated with Biotinamidocaproate N-Hydroxysuccinimide Ester (BAC) Biotin/Protein Ratio: 10-20 BAC molecules per Rabbit IgG molecule. The secondary antibody was used as 1:200 dilution in 1% BSA/ 5% goat serum/ PBS.

The secondary antibody for OPG was a biotinylated polyclonal rabbit anti-mouse (ab5761, Abcam), conjugated with Biotinamidocaproate N-Hydroxysuccinimide Ester (BAC) Biotin/Protein Ratio: 10-20 BAC molecules per Rabbit IgG F(ab')2 molecule. The secondary antibody was used as 1:500 dilution using 1% BSA/ 5% rabbit serum/ PBS.

2.7.4 Antigen Retrieval Methods

2.7.4.1 Heat Retrieval for RANKL and RANK

A number of temperatures and durations were tested. A final antigen retrieval protocol using 0.1M tri-sodium citrate (pH 6.0) at 80°C for 15 mins in a Decloaking chamber (NxGen, Biocare Medical) was used.

2.7.4.2 Trypsin Pre-treatment for OPG

The slides were incubated in 0.1% Trypsin (T7409, Sigma-Aldrich) in 0.1% $CaCl_2$ (pH 7.8) at RT for 10 mins.

2.7.5 Negative Controls

Matched immunoglobulin isotype G (IgG) was used as a negative control to ascertain and validate positive staining. The concentration of the matched negative controls was the same as the primary antibodies used. All negative controls went through the same protocol as the test group.

The negative control for both RANKL and RANK was polyclonal rabbit IgG (X0936, Dako; 1 mg/mL) at 1:200 dilution in 1% BSA/ 5% goat serum/ PBS. The OPG antibody control was normal mouse IgG (Santa Cruz Biotechnology, sc2025; 0.4 mg/mL) at a 1:80 dilution in 1% BSA/ 5% rabbit serum/ PBS.

2.7.6 Immunohistochemistry (IHC) Protocols

Full protocols for the immunochemistry procedures are given in Appendix II. All IHC procedures were carried out on the bench at room temperature (20 to 25°C) unless otherwise stated.

Prior to the procedure, all slides were clearly labeled. The slides were de-waxed in xylene for 20 minutes followed by rehydration using graded alcohol at descending concentrations of 100%, 90%, 70% and 50% for another 20 minutes. The sections were then washed with distilled water.

Antigen retrieval for OPG and the control mouse IgG, was undertaken with 0.1% trypsin for 10 minutes prior to washing with PBS for 10 minutes. The RANKL, RANK and control rabbit IgG sections underwent heat retrieval for 15 minutes at 80°C, prior to washing with PBS for 10 minutes. The sections were marked with wax pen (Dako, USA). Sections for RANKL and RANK were blocked using 20% goat serum (heat treated) (G9023, Sigma Chemical, USA) in 1% BSA/PBS for 30 minutes at RT. Sections for OPG were blocked using 20% rabbit serum (heat treated) (Sigma Chemical, USA) in 1% BSA/PBS for 30 minutes at RT.

All primary antibodies and negative controls for RANKL and RANK were diluted with 5% goat serum and 1% BSA/BPS according to the protocol. For OPG, 5% rabbit serum and 1% BSA/BPS was used. Primary antibodies or matched isotype control were applied to the sections (150 µL) and incubated at 4°C overnight in humidified chamber.

The next day the sections were washed in 3x changes of wash buffer (PBS + 1% milk powder) for 45 minutes. All sections were treated with 150 µL of secondary antibody diluted in 5% serum and 1% BSA/PBS for 30 minutes at RT in a humidified chamber. Sections were washed with of PBS (3x changes) for 25 minutes, followed by endogenous

perioxidase blocking for 10 minutes in 100 mL of 0.3% hydrogen peroxide/methanol (Merck, Germany). Sections were then washed in three changes of PBS for 15 minutes and incubated for 30 minutes at RT with 150 µL per slide of avidin-biotin horseradish perioxidase (Vectorstain Elite ABC, PK-6100, Vector Laboratories Inc, CA). The sections were washed with three changes of PBS for 15 minutes and treated with 150 µL per slide of 3, 3" diaminobenidine (DAB) chromogen (Sigma, D6190, USA) for 3 minutes. Sections were washed in distilled water (x3) for 15 minutes and stained with haematoxylin for five seconds, then washed in running tap water until clear and placed in Scott's tap water for one minute. Sections were then dehydrated in graded alcohols of 50%, 90% and 100% for two minutes each followed by xylene (x2) for five minutes each. Sections were cover-slipped (Labserv® LBS22X50-1) using DepeX (Sigman, BCBV8243), and allowed to dry.

2.7.7 Slide Scoring

The region of interest (ROI) was scored according to the strength of staining associated with osteoblasts and osteoclasts in relation to different proteins as described by Baharuddin et al., (2015). The scoring sheet is described in Appendix III.

2.8 RNA Expression Analysis

2.8.1 Tissue Collection

The third premolar site (P3) which was mesial to the first molar, was easily identified and so was used for the trephination site. Tissue collection was carried out at 4, 8 and 16 weeks at the osteotomy site (within the gap and along the periosteal/endosteal surfaces of the bone fragments). Tissue was collected from seven test sites (BioOss® + Biogide®) and seven control sites (no graft + no membrane) at each time point, with a total of 21 samples collected (see Appendix I). The tissue was placed in RNAlater™ storage solution (AM7020, Invitrogen, Thermofisher Scientific) and stored at -80°C until required.

2.8.2 Tissue Homogenisation

All instruments used for RNA extraction procedures were rinsed well with RNase AWAY™ reagent (10328-011, Ambion, Life Technologies) to remove RNases. Using tweezers the tissue sample was transferred to a homogenising tube (2 mL Precellys Lysing Kit tubes containing hard tissue grinding stainless steel beads (MK28-R, 108917-804) and placed in to the Precellys® Evolution homogenizer with a cooling attachment (Cryolys) for grinding. Homogensing cycles of 3x 7200 rpm for 30 secs, with a 20 sec pause between each cycle were used. A constant temperature of 4°C was maintained throughout the grinding process. The homogenate was recovered from the lysing kit tube with TRIzol® (500 µL, 15596026, Ambion Life Technologies), and stored at -80°C until required.

2.8.3 RNA Extraction

RNA was extracted from the homogenates using a PureLink™ RNA Mini Kit (1939052, Invitrogen). The homogenate (500 µL) was transferred to a Phasemaker® tube and incubated for five mins. Chloroform (200 µL) was then added and the tube shaken by hand for 15 secs and incubated for 2-3 mins at RT. The homogenate was then centrifuged for five mins at 14000 g at 4°C. The resulting upper aqueous phase containing the RNA was transferred to new 1.5 mL tube to which 70% ethanol (200 µL) was added and

vortexed. The sample was then transferred to a spin cartridge and centrifuged at 12000 g for 20 secs. The flow-through was discarded and the spin cartridge reinserted into the same collection tube. Wash Buffer I (700 µL) was added to the spin cartridge and centrifuged at 12000 g for 20 seconds. The flow-through was discarded and the spin cartridge reinserted in the same collection tube to which 500 µL of Wash Buffer II was added and centrifuged again at 12000 g for 20 secs. This step was repeated twice, and each time the flow-through was discarded. A final centrifugation step at 12000 g for 1 min dried the membrane and the flow-through was discarded. The spin cartridge was then transferred to a new RNAase-free 1.5 mL recovery tube and RNase free water (50 µL) added and incubated for 1 min before the RNA was to eluted from the spin cartridge by centrifugation at 12000 g for two mins. The RNA concentration was determined spectrometrically using a NanoPhotometer™ (Implen NanoPhotometer®). The purified RNA stored at -80°C until required.

2.8.4 RNA Concentration

The GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific, K0841, K0842) was used to concentrate RNA samples which were < 300 ng/µL in order to achieve the correct concentration for the next cDNA synthesis step.

Nuclease-free water (150 µL) was added to the RNA sample followed by Binding Buffer (100 µL) and ethanol (300 µL; 96-100%) and mixed well after each addition. The solution was then transferred to the purification column with collection tube and centrifuged for 60 secs a 14000 g at RT. The flow-through was discarded and the purification column place back into the collection tube and Wash Buffer I (700 µL) added. This was centrifuged again for 60 secs at 14000 g at RT and the flow-through discarded and placed back into the collection tube. Wash Buffer II (700 µL) was then added and centrifuged again at 14000 g for 60 secs and the flow-through was discarded and the purification column was replaced into the collection tube, this step was carried out twice. The purification columns were then centrifuged for one minute at 14000 g and transferred into a clean collection tube. A volume of 10 µL of nuclease-free water was added to the purification column and centrifuged for one minute at 14000 g to elute the

RNA. The concentrations of RNA in the homogenate were determined using the NanoPhotometer™ (Implen NanoPhotometer®). The purification column was discarded, and the purified RNA was stored at -20°C until required. An absorbance ratio (A260:A280) of >1.8 was considered optimum quality RNA for qPCR analysis as it signifies that the RNA is pure.

2.8.5 Complementary DNA (cDNA) Synthesis

Purified RNA was used to synthesize cDNA using the SuperScript™ IV VILO™ Master Mix kit (11766050, Invitrogen). All steps were carried out ice. The experiment was carried out in two steps that included removal of genomic (gDNA) and cDNA synthesis.

For removal of gDNA, a master mix was prepared for 25 samples using 10x eDNase Buffer (25 µL) and eDNase enzyme (25 µL). The RNA template (150 ng), the prepared master mix (2 µL) and nuclease-free water to 10 µL was placed in to a 150 µL PCR tube. The samples were then gently mixed and incubated at 37°C for 2 mins.

The cDNA synthesis step included the addition of the SuperScript™ IV VILO™ Master Mix which contained the reverse transcriptase (4 µL) and nuclease-free water (6 µL) to a final volume of 20 µL. The reaction was placed in a PCT-100® Programmable Thermal Controller (MJ Research, Waltham, USA) and incubated at 25°C for 10 mins, 50°C for 10 mins, 85°C for 5 mins and 4°C until removed. The cDNA (15 ng/µL) was stored at -20°C until required.

2.8.6 Primer Selection and Design

A number of ovine reference genes were tested for their suitability for the normalisation of cDNA input. Reference genes should be constitutively expressed (Table 2). The references gene primer sets used in this study had been previously published by Schulze et al. (2017).

Table 2: Forward and reverse primers for housekeeping genes (Gene name; Forward F or Reverse R; Ovine OV)

Name	Oligo Name	Sequence
Actin Beta	ACTBFOV	5'-GCAGATGTGGATCAGCAAGC-3'
	ACTBROV	5'-GGGTGTAACGCAGCTAACAG-3'
Ribosomal Protein L19	RPL19FOV	5'-AGCCTGTGACTGTCCATTCC-3'
	RPL19ROV	5'-ACGTTACCTTCTCGGGCATT-3'
Beta-2-microglobulin	B2MFOV	5'-CCTTGGTCCTTCTCGGGCTG-3'
	B2MROV	5'-TCTGGCGGGTGTCTTGAGTAT-3'
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta	YWHAZFOV	5'-GATGAAGCCATTGCTGAACTTGA-3'
	YWHAZROV	5'-CAGCTTCGTCTCCTTGGGTA-3'
Glyceraldehyde-3-phosphate dehydrogenase	GAPDHFOV	5'-ACAGTCAAGGCAGAGAACGG-3'
	GAPDHROV	5'-CCAGCATCACCCCACTTGAT-3'

For OPG, RANKL, Col1A1, Msx2, TIMP3 and Sp7 the ovine primer sequences were adopted from previously published research (Table 2). The primers were then mapped onto ovine genes sequence to confirm the line-up, and then re-matched to the sequences described in the different research papers. For TNFRSF11a (RANK) the primers were designed as described in Appendix IV.

The primers sequences were checked for ovine sequence homology using BLAST® software (www.ncbi.nlm.nih.gov) and ovine gene sequences retrieved from the National Center for Biotechnology Information (NCBI).

Table 3: Forward and reverse primers for genes of interest

Name	Oligo	Sequence	Reference
Osteoprotegerin	OPGFOV	5'-AGCAGCGAATCATCGACTCA-3'	Lienau et al., 2010
	OPGROV	5'-ACTGAGCCAGTTCGGGGTAA-3'	
Receptor activator of nuclear factor kappa-B	RANKFOV	5'-TCAGATGTGGTTTGTAGCGTCC-3'	Designed
	RANKROV	5'-GTTAGTGCTTTCCCTTTTTTCCT-3'	
Receptor activator of nuclear factor kappa-B ligand	RANKLFOV	5'-CCATGTCGGGGAGAGTTTTC-3'	Lienau et al., 2010
	RANKLROV	5'-AGTCAAAGGCATGGGTAGGG-3'	
Collagen, type I, alpha 1	COL1A1FOV	5'-CTGCAACCTGGATGCCATTA-3'	Lienau et al., 2010
	COL1A1ROV	5'-TTGTCCTTGGGGTTCTTGCT-3'	
Homeobox protein MSX-2	MSX2FOV	5'-CTGGTCAAACCCTTCGAGAC-3'	Craven, Rufaut, Scobie, & Nixon, 2007
	MSX2ROV	5'-AGTATCTGCCCGGTTCCTG-3'	
Tissue inhibitor of metalloproteinase 3	TIMP3FOV	5'-ACATCCACACGGAAGCCTCT-3'	Lienau et al., 2010
	TIMP3ROV	5'-TCCCACCTCTCCACGAAGTT-3'	
Transcription factor sp7	SP7FOV	5'-CAGCGGCGTGCAGTAAAT-3'	Yan et al., 2014
	SP7ROV	5'-CTGGGAACGAGTGGGAAAA-3'	

2.8.7 mRNA expression analysis

SYBR green quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were employed to determine the mRNA expression levels. A master mix for 25 samples was prepared for each of the seven genes of interest (GOI) and a selected reference gene. A master mix was prepared using SYBR Green (25 µL; 4385612, Thermo Fisher Scientific,), reverse primer (5 µL; 20 µM), forward primer (5 µL; 20 µM) and nuclease-free water (40 µL). The cDNA was diluted to 1 ng/µL using nuclease free water.

A 96-well plate was prepared with a final volume of 10 µL per well containing either 7 µL the GOI master mix or reference gene and 3 µL of cDNA. The DNA amplification was performed using the QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems,

Foster City, USA). A DNA polymerase activation step at 95°C for 20 secs, was followed by a denaturing step at 95°C for 3 secs and an annealing step at 60°C for 30 s for 40 cycles.

2.8.8 Data Analysis for Real-time Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis (qRT-PCR)

The reference gene with the most consistent Cq and melting points was selected for this study. Data analysis was performed using GraphPad Prism software V7 (GraphPad Software Inc., San Diego, USA). Changes in gene expressions were assessed as described Lienau and colleagues (2010). Cq and melting point data were used. The Cq value reflects the gene expression level, the higher the Cq value the lower the gene expression. Cq values are expressed as: $Log\ value = 2^{-\Delta Cq}$.

Chapter 3 Results

3.1 General

A total sample of 30 sheep (N=30) were included in the study. Three premolar alveolar sockets on the right side of the mandible were used for IHC, and bone from the third premolar alveolar socket was used for gene expression analysis.

There were no post-operative complications following the surgical interventions and all 30 sheep survived until euthanasia.

3.2 Immunohistochemistry

3.2.1 Decalcification and Paraffin Embedding

The alveolar sockets were decalcified in EDTA pH of 7.4. As the sockets were collected over a 16-week period, each socket was a different size, and at a different stage of healing, therefore, the time taken for the removal of the calcium from the bone varied. Generally, 4-week samples took less time to decalcify compared to the 8 and 16-week samples. (Table 4).

Table 4: Tissue Decalcification Time

	Healing Time Points (Weeks)		
	4	**8**	**16**
Time taken for decalcification (days)	112- 135	139-287	139-287
Number of blocks embedded in paraffin	45	43	41
Number of H & E slides	45	43	41

3.2.2 Tissue Section Adherence

The alveolar sockets of sheep are surrounded by lamellar bone, and this made adherence to the histology slides difficult. Tissue lifting and folding was commonly observed. To address this, slides with different adherence chemistries were investigated, as were different antigen heat retrieval temperatures and times (Appendix III). The greatest tissue adherence was achieved using Superfrost Ultra Plus® (Thermo Scientific, Cat #: J3800AMNZ) slides. The optimum heat antigen retrieval was achieved at 80°C for 15 minutes.

3.2.3 Optimal Antibody Concentrations

All IHC procedures were carried out on the bench. Optimal primary antibody concentrations are described in Table 5.

Table 5: Optimum Antibody Concentrations

Primary Antibody	Concentration, Dilution	Antigen Retrieval
RANKL	5 µg/mL, 1:200 dilution	Heat at 80°C for 15 mins
RANK	5 µg/mL, 1:100 dilution	Heat at 80°C for 15 mins
OPG	5 µg/mL, 1:200 dilution	0.1% Trypsin in 0.1% $CaCl_2$ (pH 7.8) at RT for 10 mins

3.2.4 Histological Assessment of Masson's Trichrome Staining

Trichrome staining was carried out on a total of eighteen sections, three samples/group/timepoint, (see appendix III). Presented below is one representative sample/group/timepoint.

3.2.4.1 Histological Assessment at 4-Weeks

By 4-weeks, soft tissue healing was present in both the control and test samples as evident by epithelium and connective tissue forming over the socket opening and the socket were surrounded by lamella bone. Unlike the control group (Figure 8 A&C) a bridge of woven bone can be seen over the socket opening in the test group (Figure 8 B&D). Woven bone however, was present on the lateral edges of the socket in both groups. Minimal to no Bio-Oss® particles were observed in the test samples.

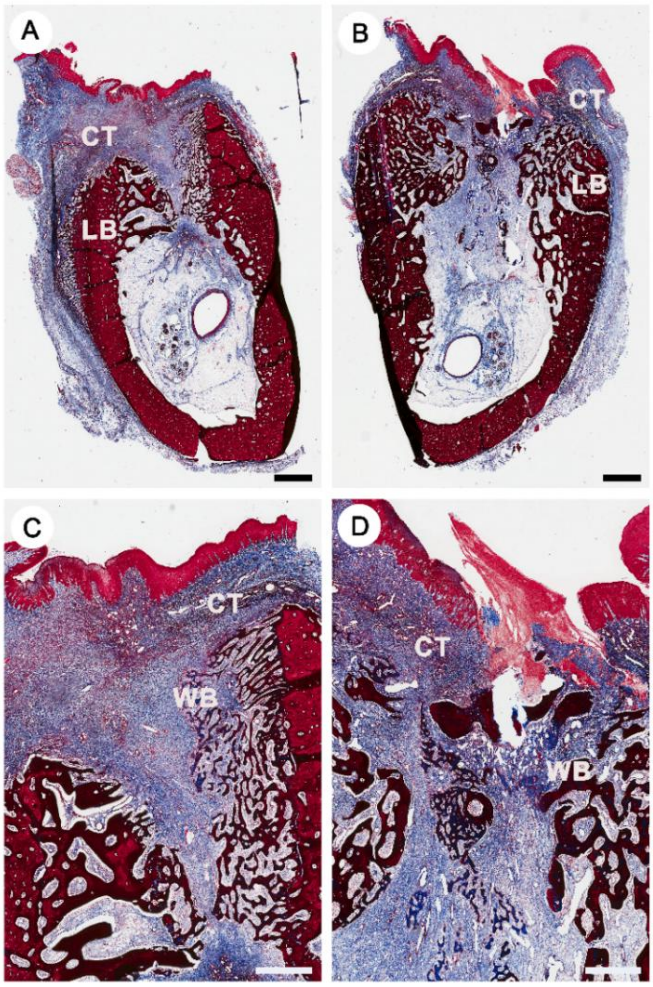


Figure 8: Masson's Trichrome staining of 4-Week samples.
(A) Control sample. (B) Test sample. (C) Central region of the dental alveolus in the control sample. (D) Central region of the dental alveolus in the test sample. Scale bar for A & B = 2 mm and C & D = 10 mm. CT - connective tissue, LB - lamella bone, WB - woven bone.

3.2.4.2 Histological Assessment at 8-Weeks

In the 8-week time point control and test samples, complete soft tissue healing was present as evident by epithelium and connective tissue over the socket opening. The sockets were surrounded by lamella bone. In the control group, there was evidence of woven bone mostly on the lateral walls of the socket (Figure 9 A&C). In the test group the socket was filled with more woven bone compared to the control group (Figure 9 B&D). Woven bone in the test group appeared to almost fill the socket. Minimal to no Bio-Oss® particles were observed in the test samples.

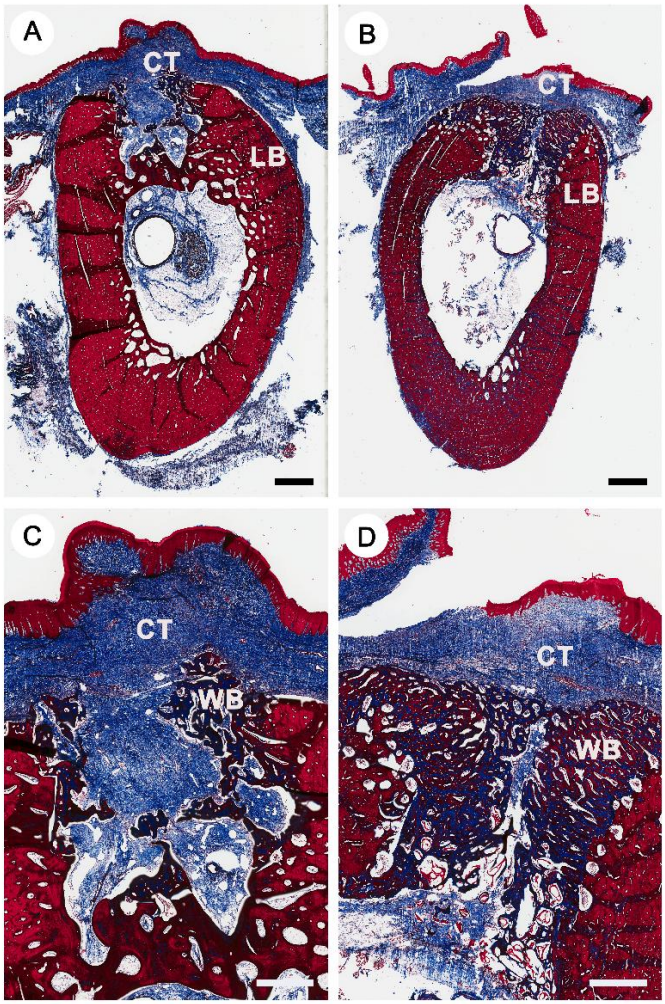


Figure 9: Masson's Trichrome staining of 8-Week samples.
(A) Control sample. (B) Test sample. (C) Central region of the dental alveolus in a control sample. (D) Central region of the dental alveolus in a test sample. Scale bar for A & B = 2 mm and C & D = 10 mm. CT - connective tissue, LB - lamella bone, WB - woven bone.

3.2.4.3 Histological Assessment at 16-Weeks

Compared to 4- and 8-week samples, there was complete soft tissue healing in both control and test samples as evident by closure of the socket opening with epithelium and underlying dense connective tissues. In both groups there was presence of woven bone that appeared more like lamellar bone. A bony bridge had formed over the socket opening in the control group with evidence of lamellar-like bone mostly on the lateral walls of the socket (Figure 10 A&C). However, in the test group the bony bridge appeared more structured with more woven bone (Figure 10 B&D). The presence of lamellar-like bone was more apparent in the test group. Minimal to no Bio-Oss® particles were observed in the test samples. Particles of Bio-Oss® appeared to be "pushed" out of the defect as healing and new bone formation progressed.

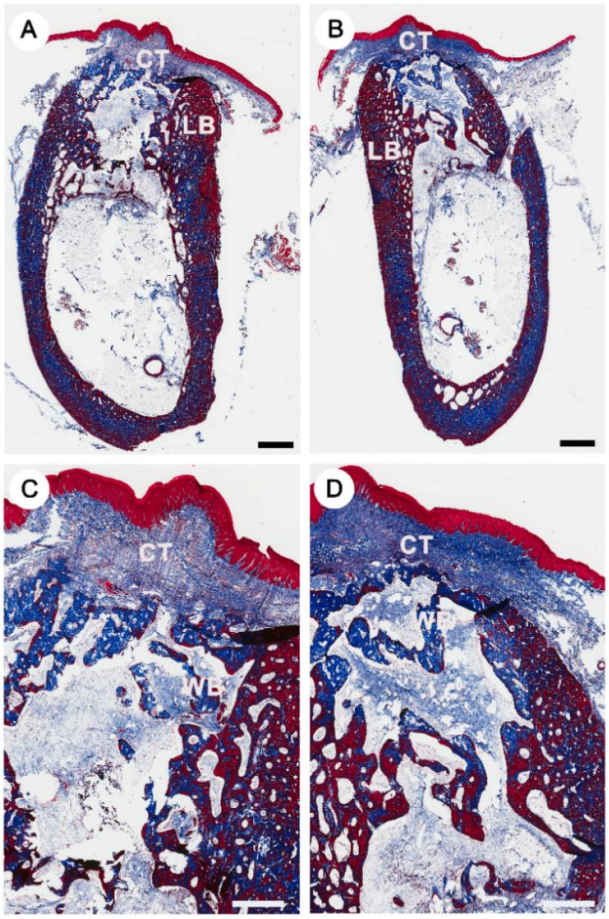


Figure 10: Masson's Trichrome staining for 16-Week samples.
(A) Control sample. (B) Test sample. (C) Central region of the dental alveolus in a control sample. (D) Central region of the dental alveolus in a test sample. Scale bar for A & B = 2 mm and C & D = 10 mm. CT - connective tissue, LB - lamella bone, WB - woven bone.

3.2.5 Immunohistochemistry Analysis

All sections were viewed before immunohistochemistry staining scores were recorded (n=18). Scores were assigned according to the strength of the staining (Appendix III). Mean scores were then calculated for each protein (Table 6). The staining scores will be interpreted in the following sections for RANK, RANKL and OPG at each healing time point.

Table 6: Summary of Immunohistochemistry

Time Points (weeks)	Cell or Tissue Type	RANKL		RANK		OPG	
		Control	**Test**	**Control**	**Test**	**Control**	**Test**
4	Osteoclasts	+++	+++	+++	++	++	++
	Osteoblasts	+++	+++	++	++	++	++
	Connective tissue	+	+	+	+	+++	+
		Control	**Test**	**Control**	**Test**	**Control**	**Test**
8	Osteoclasts	+++	+++	++	+++	+++	+++
	Osteoblasts	+++	+++	++	++	++	++
	Connective tissue	+	+	+	+	+++	+++
		Control	**Test**	**Control**	**Test**	**Control**	**Test**
16	Osteoclasts	+++	+++	+++	+++	+++	+++
	Osteoblasts	+++	+++	+	+	+	+
	Connective tissue	+	+	+	+	+	+

Slide Scoring. For RANK/ RANKL positive staining were scored as + (1-2 positive osteoclasts per section), ++ (3-10 positive osteoclasts per section), +++ (>10 positive osteoclasts per section). ND, no positive staining detected. For RANKL/ OPG positive staining were scored as + (small number of positive cells), ++ (moderate number of positive cells) and +++ (large number of positive cells).

3.2.5.1 IHC Assessment for RANKL

Strong immunopositive staining for RANKL was observed on osteoblasts and osteoclasts both in the control and test groups across all time points. At 4 weeks RANKL staining on osteoblasts was found around new bone. At 8 and 16 weeks, staining of RANKL on osteoclasts was more apparent around remodelling bone on the edges of the socket. Localisation of RANKL in the connective tissues appeared to be isolated and mild across both groups and all time points (Figure 11 A–F). No positive staining was observed in the negative controls for RANKL (Figure 11 G&H). Minimal to no Bio-Oss® particles were observed in the test samples.

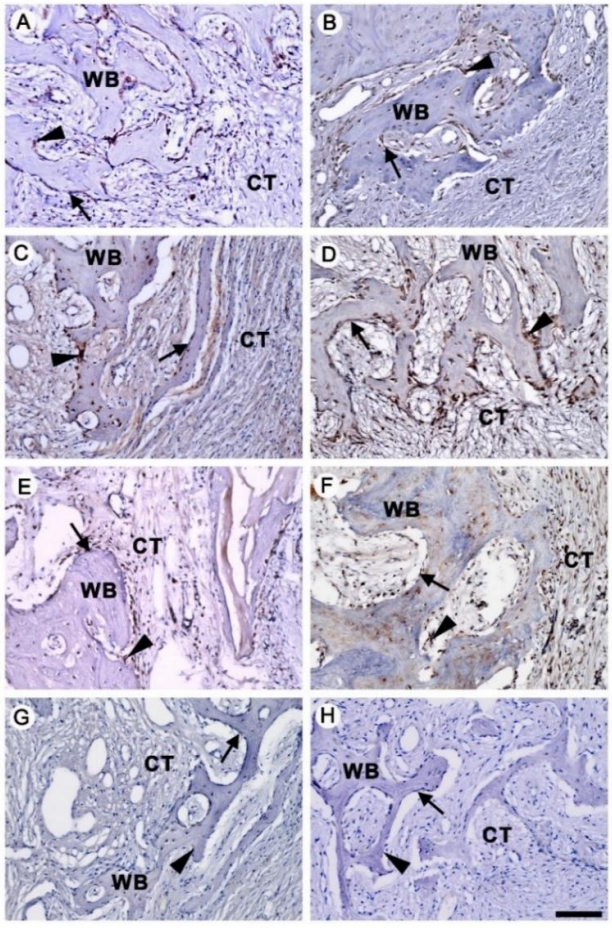


Figure 11: Immunohistochemical localisation of RANKL and Rabbit IgG (negative control) at coronal edge of the dental alveolus.
Strong immunopositive staining for RANKL (brown colouration) was observed on osteoblasts and osteoclasts both in the control and test groups. Bio-Oss® was not observed in the test samples. (A) 4-weeks control (B) 4-weeks test (C) 8-weeks control (D) 8-weeks test (E) 16-weeks control (F) 16-weeks test (G) control rabbit IgG. (H) test rabbit IgG. CT - connective tissue, WB - woven bone, black arrow - osteoblasts, black arrowhead - osteoclasts. Scale bar = 100 µm.

3.2.5.2 IHC Assessment for RANK

At the 4-week time point, moderate staining of RANK was observed in association with osteoblasts and osteoclasts in both the control and test groups. At 8 weeks both groups showed moderate immunopositivity on osteoblasts and by 16 weeks only mild staining could be seen. Moderate osteoclast RANK staining was observed in the control group at 8 weeks however, strong staining of RANK was associated with osteoclasts in the test groups at 8 and 16 weeks. Localisation of RANK in the connective tissues appeared to be isolated and mild in both groups and all time points (Figure 12 12 A–F). No positive staining was observed in the negative controls for RANK (Figure 12 G&H). Minimal to no Bio-Oss® particles were observed in the test samples.

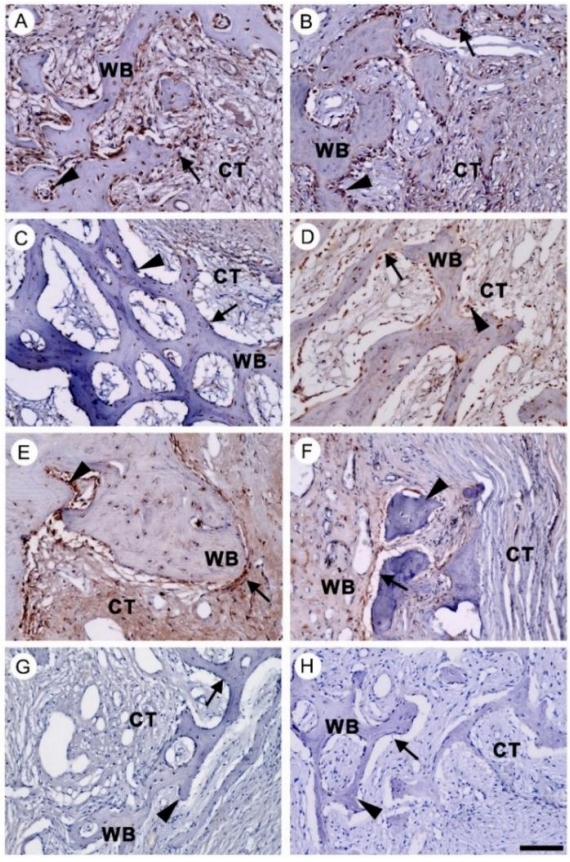


Figure 12: Immunohistochemical localisation of RANK and Rabbit IgG (negative control) at coronal edge of the dental alveolus.
Moderate immunopositive staining for RANK (brown colouration) was observed on osteoblasts and osteoclasts both in the control and test groups. Bio-Oss® was not observed in the test samples. (A) 4-weeks control. (B) 4-weeks test. (C) 8-weeks control. (D) 8-weeks test (E) 16-weeks control. (F) 16-weeks test. (G) control rabbit IgG. (H) test rabbit IgG. CT - connective tissue, WB - woven bone, black arrow - osteoblasts, black arrowhead - osteoclasts. Scale bar = 100 µm.

3.2.5.3 IHC Assessment for OPG

Localisation of OPG in the connective tissues appeared diffuse and was stronger during the early healing periods and gradually decreased over time. At 4 weeks moderate staining of OPG was observed on osteoblasts and osteoclasts both in the control and test groups (Figure 13 A–B). OPG staining on osteoblasts was moderate at 8 weeks and mild at 16 weeks in both groups. However, strong staining of OPG was associated with osteoclasts in both the control and test groups at 8 and 16 weeks of healing (Figure 13 C–F). No positive staining was observed in the negative controls for OPG (Figure 13 G&H). Minimal to no Bio-Oss® particles were observed in the test samples.

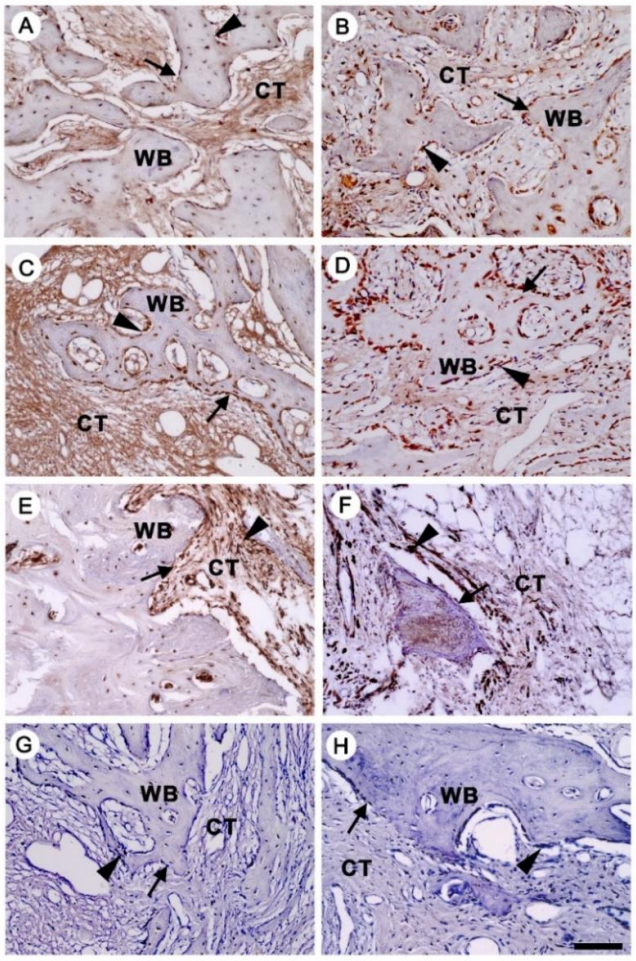


Figure 13: **Immunohistochemical localisation of OPG and Mouse IgG (negative control) at coronal edge of the dental alveolus**
Strong immunopositive staining for OPG (brown colouration) was observed on connective tissue both in the control and test groups. Bio-Oss® was not observed in the test samples. (A) 4-weeks control. (B) 4-weeks test. (C) 8-weeks control. (D) 8-weeks test. (E) 16-weeks control. (F) 16-weeks test. (G) control mouse IgG. (H) test mouse IgG. CT - connective tissue, WB - woven bone, black arrow - osteoblasts, black arrowhead only - osteoclasts. Scale bar = 100 μm.

3.3 Gene Expression Analysis

Gene expression analysis was carried out at three time points, 4, 8 and 16 weeks. Third premolar sockets (P3) treated with Bio-Oss® and Bio-Gide® (test) or untreated for natural unassisted healing (control) were compared. A total of 21 tissue samples were collected, with 18 samples used for RT-qPCR mRNA expression analysis.

Tissue from the sheep alveolus is generally very dense, therefore it was decided to take two of the 21 samples to determine if using a cryo-grinder (Freezer/ Mill® 6670, SPEX SamplePrep) could effectively homogenise the tissue for RNA extraction. It was however, found that due to the large size of the cryo-grinder sample holder, the small amount of fine power it produced could not be recovered effectively. To overcome this, an automated homogeniser (Precellys® Evolution, Bertin Technologies, France) designed for smaller samples was used to homogenise the tissue.

A total of 18 samples were used for gene expression analysis, three test and three controls for each of the time points (n=6). Three additional control group samples were collected for reference gene validation, out of which two were then also included in the final experiments.

3.3.1 Quality and Quantity of Purified Sheep total RNA

Spectroscopic analysis as determined by the A_{260}/A_{280} ratio showed the purified RNA to be within the acceptable range (1.8 – 2.0). Each of the 21 samples yielded total RNA of between 28 – 320 µg (Table 7).

Table 7: The Quality and Quantity of RNA from Alveolar Sockets in Sheep

Unique Number	Sheep Number	Time Point (weeks)	Test group	A260	A260/ A280	RNA Concentration (ng/µL)	Total RNA
1	597‡	4	C	0.045	1.704	18.4	920
2	432	4	B	0.198	1.756	60.4	3020
3	586*	4	C	0.320	1.899	128.0	6400
4	441	4	B	0.149	1.886	59.6	2980
5	583‡	4	C	0.028	1.556	11.2	560
6	428	4	B	0.091	1.820	36.4	1820
7	595	4	C	0.081	1.745	32.8	1640
8	440‡	8	C	0.047	1.750	19.6	980
9	589	8	B	0.085	1.771	34.0	1700
10	434†	8	C	0.031	1.833	13.2	660
11	598‡	8	B	0.035	1.667	14.0	700
12	426*	8	C	0.152	1.831	60.8	3040
13	438	8	B	0.158	1.847	62.8	3140
14	450	8	C	0.169	1.888	67.2	3360
15	430	16	C	0.324	1.878	129.0	6450
16	599	16	B	0.096	1.846	38.4	1920
17	584*	16	C	0.119	1.872	47.2	2360
18	585‡	16	B	0.061	1.848	24.4	1220
19	442†	16	C	0.053	1.828	21.2	1060
20	427‡	16	B	0.048	1.778	19.2	960
21	425	16	C	0.095	1.843	37.6	1880

† Samples not included due to them being lost in the cryo-grinder homogenising procedure * Samples used to validate reference gene ‡ RNA samples which required concentration (<25ng/µL). As noted in the Methods section, only treatments B (Bio-Oss® + Bio-Gide®) and C (un-grafted control) were analysed. Treatment A was analysed is a separate study (not reported here).

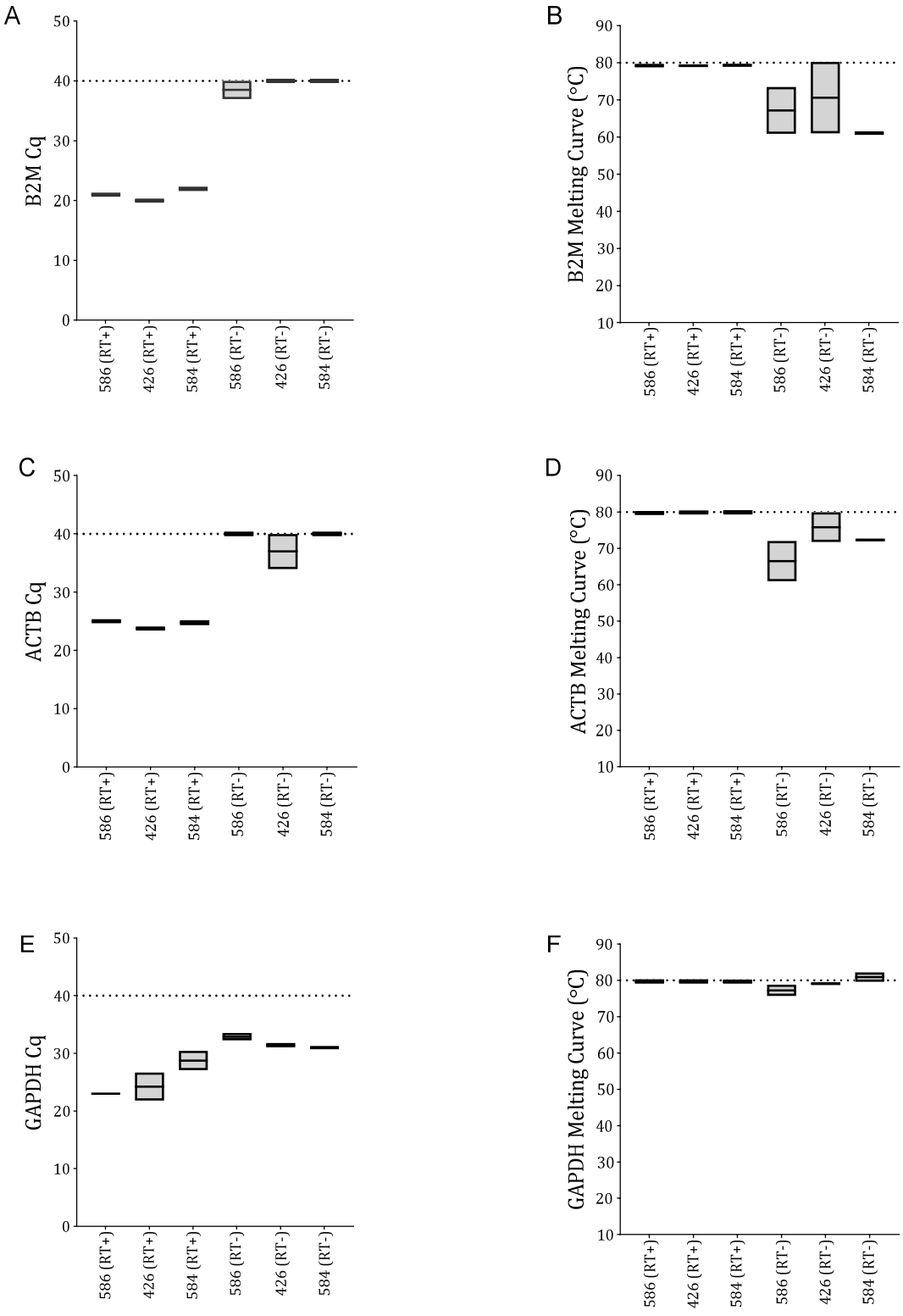
3.3.2 Reference Gene Validation - SYBR Green qPCR assays

The suitability of the reference genes were assessed, based on Cq values and melting curves using three tissue samples.

The primer set for B2M resulted in low and consistent Cq values for the RT+ samples and no detectable product amplification for the RT- samples (indicating no gDNA was present in the cDNA template used for the SYBR Green assay) (Figure 14A). Melting temperature values were also consistent, indicating a similar sized product was amplified for each RT+ cDNA template and RT- samples were indictive of no product being amplified of a correct size (Figure 14B).

For the ACTB primer set, high and consistent Cq values for the RT+ samples were observed. The RT- samples showed no detectable product amplification indicating absence of gDNA in the cDNA template (Figure 14C). Melting temperature values for ACTB was also consistent which indicated that similar sized product was amplified for each RT+ cDNA template and RT- samples indicated that no products of the correct size were amplified (Figure 14D).

The GAPDH, RPL19 and YWHAZ primer sets failed to amplify the correct product as evident by inconsistent Cq values for the RT+ samples (Figure 14E, G, I). These three primer sets detected product amplification in the RT- samples, indicating incorrect product amplification. The melting temperatures for GAPDH, RPL19 and YWHAZ were similar for RT+ and RT- samples indicating amplification of incorrect product (Figure 14F, H, J).


A
B2M Cq
B
B2M Melting Curve (°C)
C
ACTB Cq
D
ACTB Melting Curve (°C)
E
GAPDH Cq
F
GAPDH Melting Curve (°C)
586 (RT+)
426 (RT+)
584 (RT+)
586 (RT-)
426 (RT-)
584 (RT-)

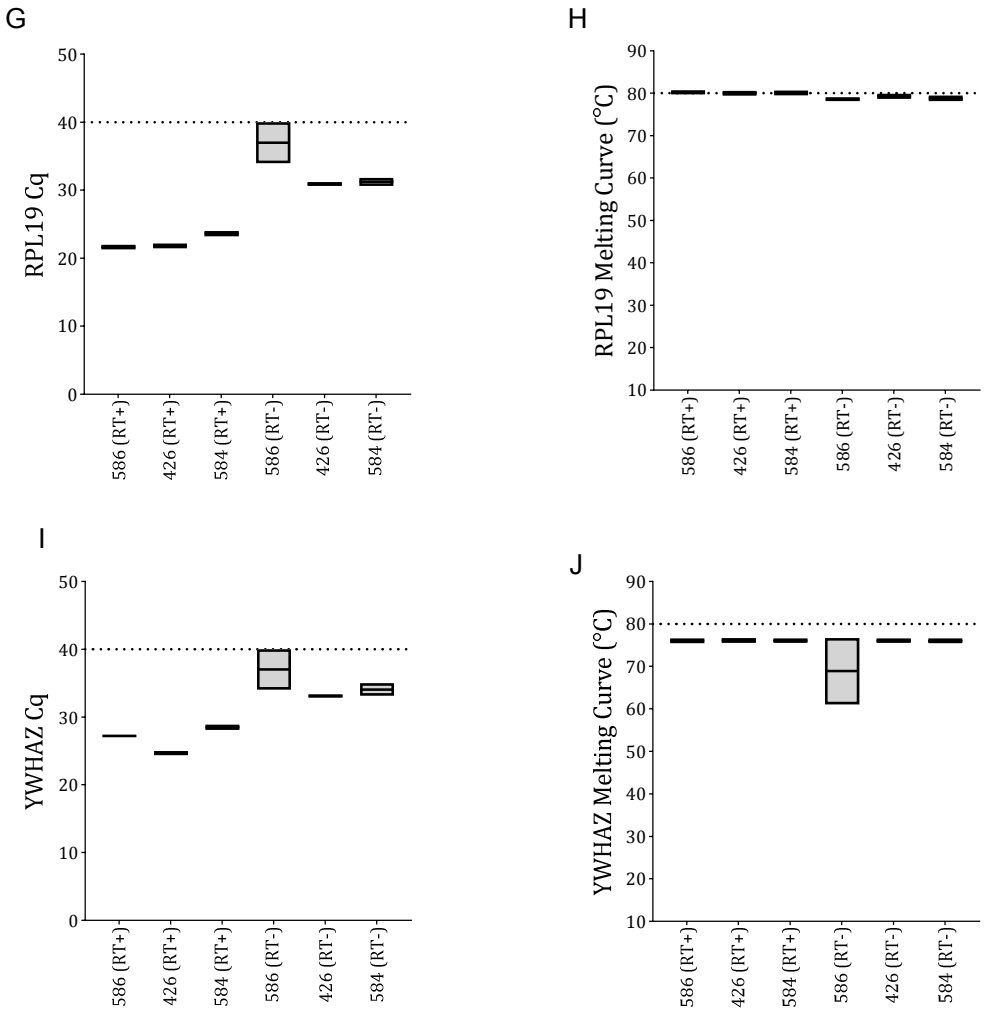


Figure 14: Cq and Melting Curve for the Reference Genes. RT (+) and RT (-) are compared. The dotted lines represent the upper limit of gene detection.

The most consistent and abundant expression was observed for B2M with little variation in the expression levels between the control and test groups over the three time points (Figure 15).

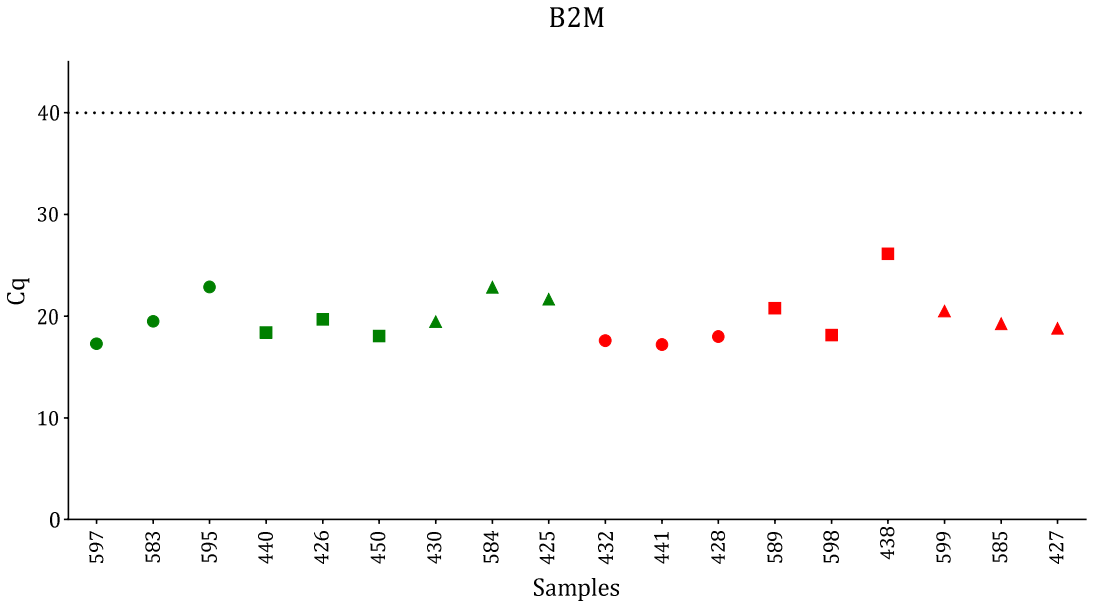


Figure 15: B2M mRNA expression.
Cq values across the 18 samples were consistent. Control samples are shown in green and test in red with 4-weeks (dots), 8-weeks (squares) and 16-weeks (triangles). The dotted line represents the upper limit of gene detection. Data shown as mean (SE).

3.3.3 Natural Healing (Control) and Healing with Bio-Oss® and Bio-Gide® (Test) - mRNA Expression

All seven osteogenic genes examined were detected in both control and test groups at each of the three time points.

3.3.3.1 RANK/RANKL/OPG mRNA Expression

RANK mRNA expression increased steadily over the three healing time points for both the control and test groups. At 4 weeks the level of RANK mRNA expression was significantly lower in the test group compared to the control group (-4.26-fold test/control; *p=0.02*). However, by 8 weeks the level of RANK expression in the test group had increased compared to the control group (4.54-fold test/control; *p=0.42*). By 16 weeks mRNA expression of RANK decreased in the test group compared to the control group (-2.19-fold test/control; *p=0.28*). (Figure 16)

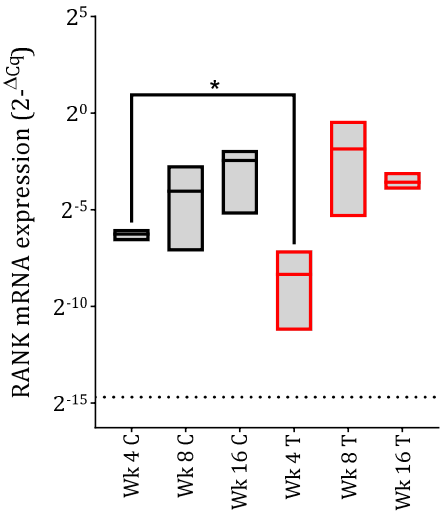


Figure 16: RANK mRNA Expression.
Expression levels are shown for each time point over the 16-week experiment. Control samples are shown in black (n=3 sheep/time point) and test samples are shown in red (n=3 sheep/time point). The dotted line represents gene undetected. Bars represent max and min with a line at the mean. *p-value < 0.05.

The expression of RANKL was similar in the control and test groups over all three time points. RANKL mRNA expression levels at 4 weeks for both groups were lower than 8 and 16 weeks (Figure 17, Table 5). Overall, for both the control and test groups, RANKL expression level increased at 8 weeks and remained steady at 16 weeks. None of the comparisons were statistically significant *p>0.05.*

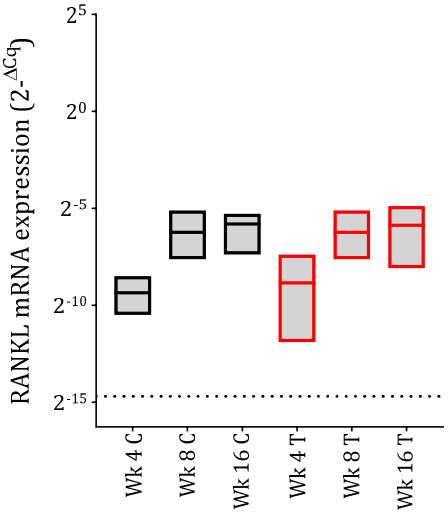


Figure 17: RANKL mRNA Expression.
Expression levels are shown for each time point over the 16-week experiment. Control samples are shown in black (n=3 sheep/time point) and test samples are shown in red (n=3 sheep/time point). The dotted line represents gene undetected. Bars represent max and min with a line at the mean.

For the test group there was a steady increase in OPG expression over the three time points. The expression level of OPG was higher in the test than the control groups at 4 weeks (3.00-fold test/control; *p=0.07)*, however, by 8 weeks, expression level was similar in both groups. At 16 weeks OPG mRNA expression markedly increased in the test group compared to the control (150-fold test/control; (*p=0.38*). In the control group OPG expression level increased at 8 weeks and then decreased by 16 weeks (Figure 18, Table 8). None of the comparisons were statistically significant *p>0.05.*

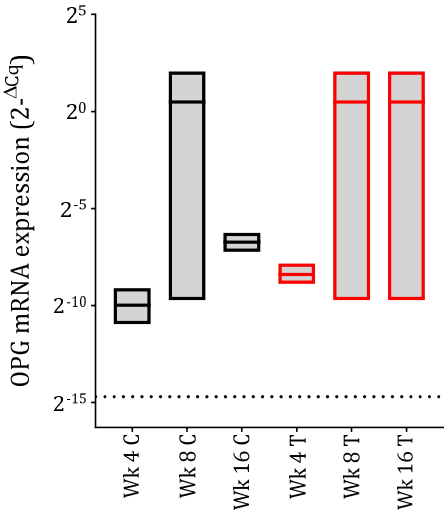


Figure 18: OPG mRNA Expression.
Expression levels are shown for each time point over the 16-week experiment. Control samples are shown in black (n=3 sheep/time point) and test samples are shown in red (n=3 sheep/time point). The dotted line represents gene undetected. Bars represent max and min with a line at the mean.

3.3.3.2 Expression of Msx2 and Sp7

At 4 weeks Msx2 expression level increased in the control group compared to the test group (-5.46-fold test/control; *p=0.11*). However, by 8 weeks the expression levels increased in the control group compared to the test group (1.5-fold test/ control; *p=0.73*). By 16 weeks mRNA expression of Msx2 increased again in the control group compared to the test (-1.82-fold test/control), (*p=0.36*). (Figure 19A, Table 8). Although not significant, there was a marked increase in Msx2 expression in the test group from 4 weeks to 8 weeks. None of the comparisons were statistically significant *p>0.05.*

Sp7 mRNA expression increased over time in test and control samples. At both 4 and 8 weeks, the Sp7 expression in the test group was increased compared to the control group (1.55-fold test/control; *p=0.34* and 1.79-fold test/control; *p=0.66,* respectively). Expression level of Sp7 significantly decreased in the test group compared to the control at 16 weeks (-2.89-fold test/control; *p=0.04*) (Figure 19B, Table 8). Initial lower expression levels of Sp7 compared to MSX2 were found, with similar levels for the control and test groups.

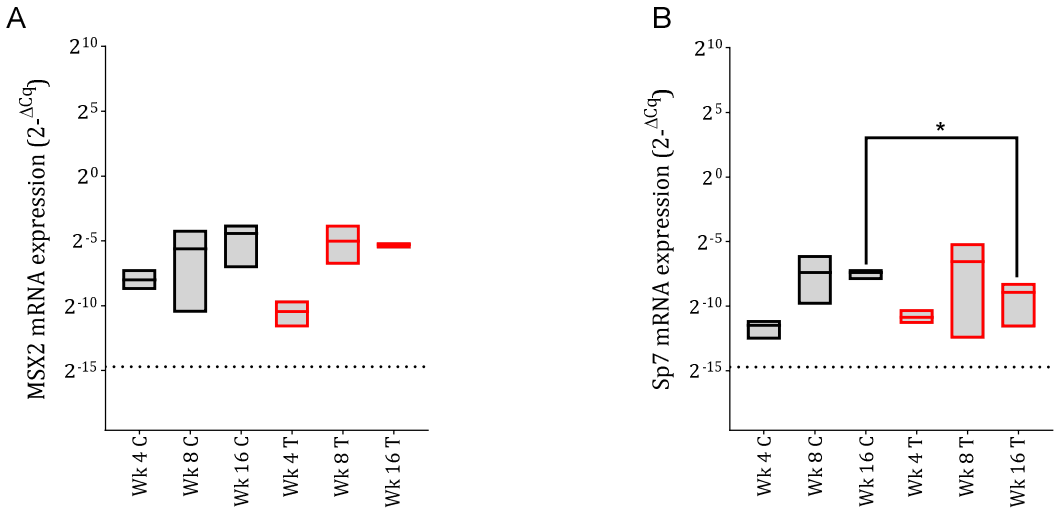


Figure 19: (A) Msx2 and (B) Sp7 mRNA Expression.
Expression levels are shown for each time point over the 16-week experiment. Control samples are shown in black (n=3 sheep/time point) and test samples are shown in red (n=3 sheep/time point). The dotted line represents gene undetected. Bars represent max and min with a line at the mean. *p-value < 0.05.

3.3.3.3 Expression of Col1A1 and TIMP3

At 4 weeks expression level of Col1A1 was similar in the control and test groups. By 8 weeks Col1A1 expression level was increased for both groups, however, the expression in the test group was higher than the control group (3.04-fold test/control; *p=0.50*). By 16 weeks Col1A1 expression level increased in control group compared to the test group (-1.64-fold test/control *p=0.41*). (Figure 20A). None of the comparisons were statistically significant *p>0.05.*

TIMP3 expression levels were generally lower compared to the Col1A1 expressions over all time points in both groups. At 4 and 8 weeks TIMP3 expression level was higher in the test group compared to the control group (6.79-fold test/control *p=0.29;* 6.05-fold test/control *p=30*, respectively). By 16 weeks expression level of TIMP3 increased in the control group compared to the test group (-2.98-fold test/ control; *p=0.18*) (Figure 20B, Table 8). None of the comparisons were statistically significant *p>0.05.*

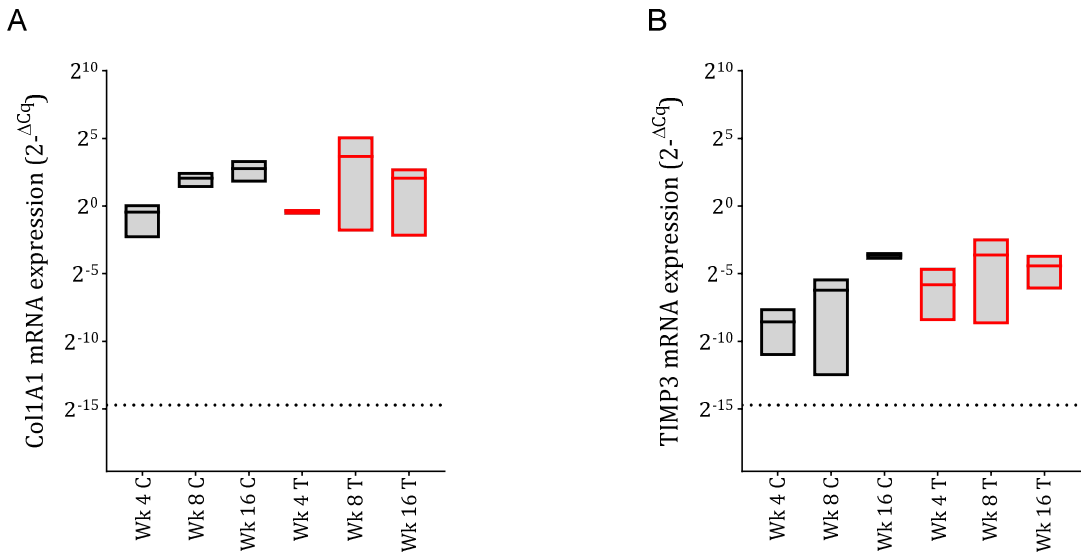


Figure 20: (A) Col1A1 and (B) TIMP3 mRNA Expression.
Expression levels are shown for each time point over the 16-week experiment. Control samples are shown in black (n=3 sheep/time point) and test samples are shown in red (n=3 sheep/time point). The dotted line represents gene undetected. Bars represent max and min with a line at the mean.

3.3.4 Differential mRNA expression (Test Group/Control Group)

Both RANK and MSX2 expression level was decreased in the test group when compared to the control group at 4 and 16 weeks, and this was statistically significant for expression of RANK (*p=0.02)*. At 8 weeks both RANK and MSX2 expression level increased in the test group compared to the control group, although not significantly.

In comparison to the control group, RANKL expression level increased in the test group at 4 weeks, remained the same at 8 weeks and decreased by 16 weeks, however, these changes were not statistically significant. OPG expression level in the test group was increased at the 4 and 16 weeks compared to the control group, although this was not statistically significant; there was no change at 8 weeks.

Col1A1, TIMP3 and Sp7 expression levels increased in the test group at 4 and 8 weeks compared to the control group and decreased at 16 weeks. Apart from the expression of Sp7 at 16 weeks (*p=0.04)*, none of the other comparisons were statistically significant *p-value* > 0.05 (Table 8).

Table 8: Differential mRNA expression

Genes	4 Weeks		8 Weeks		16 Weeks	
	Fold-difference (test/control)	***P value***	**Fold-difference (test/control)**	***P value***	**Fold-difference (test/control)**	***P value***
RANK	-4.26	0.02*	4.54	0.43	-2.19	0.28
RANKL	1.44	0.75	1.00	>0.99	-1.04	0.96
OPG	3.00	0.07	1.00	>0.99	150.53	0.38
Col1A1	1.05	0.90	3.04	0.50	-1.64	0.41
TIMP3	6.67	0.29	6.05	0.30	-2.89	0.18
Sp7	1.55	0.34	1.79	0.66	-2.89	0.04*
Msx2	-5.46	0.11	1.53	0.73	-1.82	0.36

**p-value* <0.05

Chapter 4 Discussion

4.1 Overview

Histological assessment of alveolar sockets preserved with the particulate xenograft Bio-Oss® and the collagen membrane Bio-Gide® appeared to show more woven bone in comparison with natural healing 16-weeks post-surgery, however, it should be noted that our study did not quantify the amount of new bone formation. mRNA expression of seven key osteogenic markers was determined using qPCR assays. The hypothesis was disproven; although the key markers of osteogenesis, RANK, RANKL and OPG were present and mRNA for key regulators of osteoblast differentiation were expressed in healing tooth sockets, grafting with bone xenograft (Bio-Oss®) and resorbable membrane (Bio-Gide®) did not change this in any marked fashion. Test (grafted) and control (un-grafted) groups were comparable at all time points, except for RANK expression (lower in test versus control group at 4 weeks $p=0.02$) and Sp7 mRNA expression (lower in test versus control at 16 weeks, $p=0.04$). This result was confirmed by immunohistochemical analysis for protein expression of RANK/ RANKL/ OPG, which were found to be comparable at all time points.

4.2 RANK, RANKL and OPG Axis

To the best of the investigator's knowledge, this is the first large animal model to evaluate RANK, RANKL and OPG expression during natural and grafted tooth socket healing. The balance between bone formation and resorption is maintained by the RANK, RANKL and OPG axis. RANKL is expressed by osteoblasts and when it binds to its receptor RANK on the surfaces of pre-osteoclasts the process of osteoclastogenesis is initiated leading to bone resorption (Figure 21). The action of RANKL can be counteracted via OPG that binds to RANKL and downregulates the action of RANK and therefore preventing bone resorption (Boyle et al., 2003).

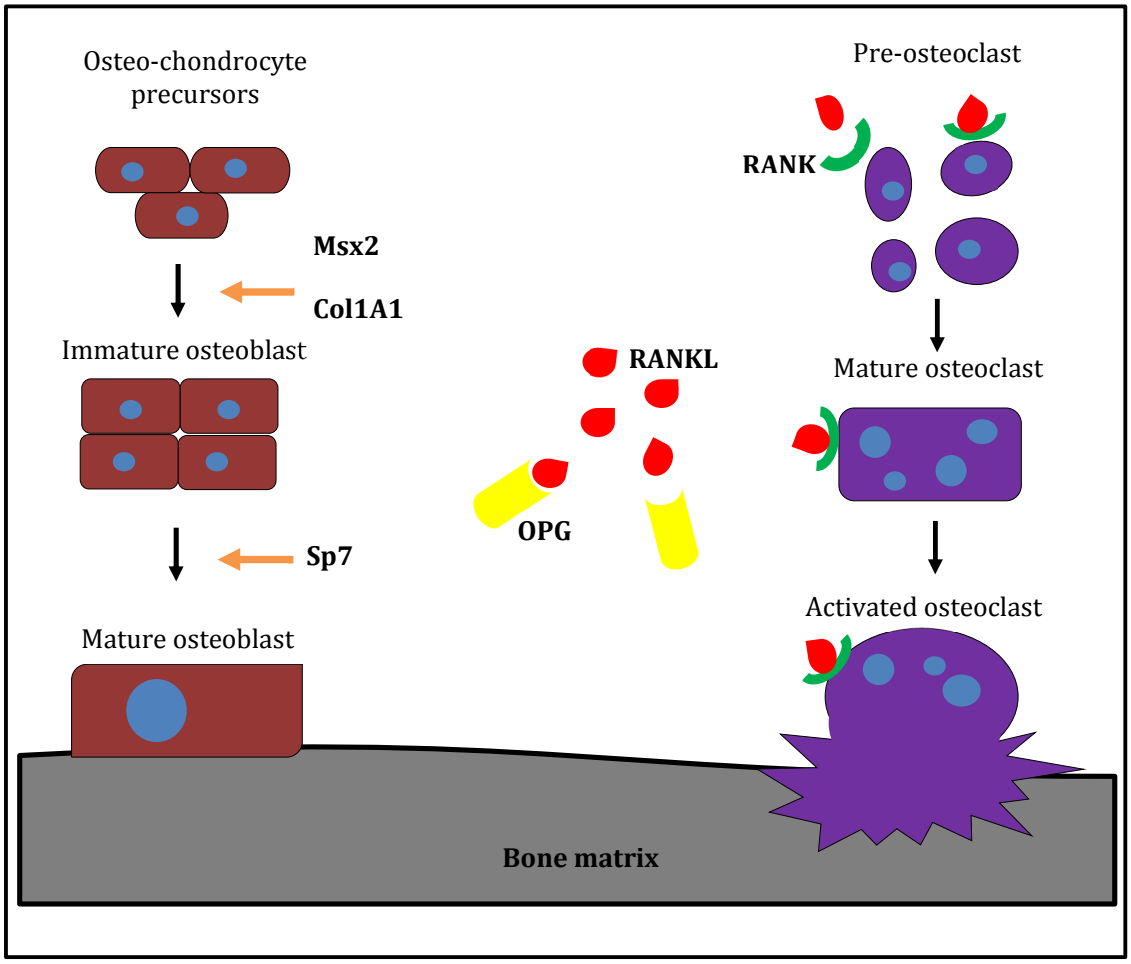


Figure 25: Schematic diagram showing the role of osteogenic markers in bone formation and resorption.

RANKL is essential for osteoclast maturation. RANKL binds to the receptor RANK on pre-osteoclasts, stimulating the differentiation and activation of osteoclasts and thus controlling the modelling and remodelling of bone. OPG is an antagonistic endogenous receptor that binds and removes RANKL and inhibits osteoclastogenesis, preventing excessive bone loss. Msx2 and Col1A1 are essential for osteoblast differentiation and Sp7 is an osteoblast-specific factor and is induced directly by Msx2. Msx2, Col1A1 and Sp7 overall regulate osteoblast differentiation and bone formation. Adapted from (Alves, 2012; Lampropoulos et al., 2012).

In the present study the expression of RANK, RANKL and OPG was comparable in the control and test groups over all time points, suggesting that the rate of osteoclastic activity is similar in the spontaneously healing and grafted socket. In both groups moderate staining of RANK was evident on osteoclasts. In the present study osteoclasts were identified based on their morphology, however, tartrate-resistant acid phosphate (TRAP) staining would have been useful for confirming this. Similar findings for protein expression of RANK and RANKL have been reported by Canullo and colleagues (2016)

after four months of healing following ridge preservation in human extraction sockets using magnesium-enriched hydroxyapatite (MgHA) graft. In the present study, the protein expression of OPG was similar in control and test groups over time, and in both groups strong staining of OPG was associated with osteoclasts. Canullo and colleagues (2016) also reported no changes in OPG protein expression in grafted sockets from baseline to four months of healing. The presence of OPG, which is a decoy receptor for RANKL (Boyle et al., 2003), inhibits the binding of RANKL to RANK, resulting in reduced osteoclastogenesis.

A clinical study of histological healing and gene expression in maxillary sinus floor augmentation (MSFA) with Bio-Oss®, compared with healed posterior alveolar ridge, found no difference in RANKL and OPG mRNA expression after six months (Suwanwela et al., 2017). Histologically the MSFA region showed similar healing to the present study with new bone and fibrous tissue formation, however, this was not compared to naturally healed tooth sockets. Overall, histological findings have shown xenografts are replaced with new bone without a detectable associated change in gene expression. In a similar clinical study, sinus augmentation was carried using either autologous bone particles or MgHA. At five months of healing, decreased mRNA expression of RANKL was present in the MgHA group with comparable expression of OPG in both groups. The lower RANKL/ OPG ratio indicated reduced osteoclastogenic activity in the presence of grafting materials as evident by comparable levels of bone volume (Crespi et al., 2009). In the present study, the grafted sockets showed decreased expression of RANKL compared to the control group.

A rabbit study has been undertaken with implants placed in the tibia and the gaps between the implant and bone either filled with blood clot, Bio-Oss® or Bio-Oss® collagen (Dos Santos et al., 2016). Histological and gene expression analysis were carried out at 15, 30 and 60 days. OPG mRNA expression increased over time with higher expression in the Bio-Oss® group at 60 days. For RANKL, the mRNA expression was higher in the blood clot group in the early phases of healing, which could indicate higher resorption.

In our study, OPG mRNA expression was markedly higher, although not significantly, in the Bio-Oss® grafted test group at 16 weeks. As OPG is a regulator of osteoclasts

differentiation and activity, we might expect to see a reduction in bone resorption with up-regulated bone formation in the presence of Bio-Oss® however, this was not reflected in the immunohistochemical images of OPG protein expression. The findings of the present study are not consistent with the study by Dos Santos colleagues (2016) who reported higher protein expression of OPG and RANKL in Bio-Oss® grafted group compared to natural healing. The mRNA and protein expression of RANKL in the present study were very similar in the control and test groups, suggesting that levels of resorption were comparable in both groups, given that RANKL expression is related to activation of bone resorption (Dos Santos et al., 2016)

In summary, the expression of RANK/RANKL/OPG was not significantly different in the spontaneous healing compared to the Bio-Oss® and Bio-Gide® grafted sockets. Osteoclastic activity was evident with the detection of RANKL and RANK mRNA and protein expression indicative of bone resorption occurring in both groups over time. When RANKL and RANK expression are considered collectively, there appears to be more bone resorption in the test group suggesting decreased healing at 8 weeks, however by 16 weeks the levels were comparable. Bone formation activity as determined by OPG mRNA and protein expression, was also shown to be comparable in both groups.

The histological findings of the present study are similar to a number of previously published studies (Canullo et al., 2016; Dos Santos, et al., 2016; Canciani et al., 2017; Suwanwela et al., 2017); the current study however, has endeavoured to also quantitatively analyse the level of mRNA expression of key osteogenic regulators.

4.3 Gene Expression Analysis - Osteogenic Markers Sp7, MSX2 Col1A1 and TIMP3

Sp7 is the only osteoblast-specific transcription factor identified to date and is essential for osteoblast differentiation and thus bone formation (Jin et al., 2019) (Figure 21). In our study, Sp7 mRNA expression increased over time in both the control and test groups, but significantly decreased at 16 weeks in the test group. This decreased expression during the late phase of healing in the grafted socket suggests that bone formation was

reduced as the result of decreased osteoblast differentiation. In the present study, Sp7 mRNA expression was higher in the test group at 4 and 8 weeks compared to the control group, although this was not statistically significant. It is possible that osteogenesis starts earlier in tooth sockets filled with Bio-Oss® compared to naturally healing sockets, before slowing down at 16 weeks, however our study lacked the power to determine this. Tan and colleagues (2019) measured the level of Sp7 mRNA expression in a socket filled with Bio-Oss® plus hypoxia-inducible factor (HIF)-1α protein versus Bio-Oss® alone, in a dog model. At 12 weeks of healing the mRNA expression of Sp7 for both grafted sockets was higher than the naturally healing sockets (Tan et al., 2019).

Msx2 is a marker for osteoblast differentiation and hence plays an essential role in osteogenesis (Ichida et al., 2004). Msx2 mRNA expression increased over all time points in both the control and test groups. Msx2 mRNA expression was lower in the sockets grafted with Bio-Oss® compared to naturally healing sockets at 4 and 16 weeks, suggesting that more osteogenic activity was present in the natural healing sockets; however, these results were not statistically significant. The role of Msx2 in bone healing is well established (Ichida et al., 2004; Matsubara et al., 2008). However, information on the role it plays in the healing of the tooth socket following tooth extractions is scarce.

As Sp7 expression is induced directly by Msx2 (Matsubara et al., 2008), when the expression levels of Sp7 and Msx2 together are considered, the expression level of these markers of osteogenesis is higher in the natural healing sockets compared to sockets grafted with Bio-Oss® and Bio-Gide®.

ColA1 is a marker for extracellular matrix production and is expressed at the early stages of bone healing and is considered an osteogenic differentiation marker (Berendsen et al., 2014; Cardoso et al., 2011; Stover & Verrelli, 2011). The Col1A1 mRNA expression levels in the present study increased steadily in the natural healing socket. In the sockets grafted with Bio-Oss® and Bio-Gide® the Col1A1 mean mRNA levels increased in the early healing phases and remained high in the later healing phases.

TIMP3 plays a role in the inhibition of ECM remodelling (Biguetti et al., 2018). TIMP3 mRNA expression in the present study steadily increased over time in the control group, however in the test group TIMP3 was high over weeks 4 to 16 weeks. The healing pattern

becomes more apparent when Col1A1 and TIMP3 mRNA expression are considered collectively. Col1A1 is a marker of ECM and TIMP3 which are associated with remodelling; both increased in the early phase of healing in the grafted sockets. It can be hypothesised that, despite being not statistically significant, ECM may be laid down earlier in the grafted sockets due to the scaffolding role of Bio-Oss®.

4.4 Use of Sheep as a Large Animal Model

The sheep model has been extensively used for orthopaedic research (Lippuner et al., 1992; Martini et al., 2001; Pearce et al., 2007). Approximately 9-12% of all animals used in research are sheep (Martini et al., 2001). Bone remodelling activities in sheep have reported to be similar to humans, suggesting that this model is useful for extrapolating in vivo data to clinical settings (Martini et al., 2001). The micro- and macrostructure of bone in sheep is reported to be similar to humans with the age of sheep playing a key role in the timing of events in the remodelling process (Nafei et al., 2000; Pearce et al., 2007). The sheep used in the present study were all 3-4 years old.

Bone healing in the presence of grafting material in sheep mandible was initially reported by Gatti et al. (1990). The sheep mandible has been used previously to examine healing of bony defects at 8 and 16 weeks and again at 12 months (Gatti & Zaffe, 1991a, 1991b). Despite having a small sample size of 2-6 sheep, these initial studies validated the sheep mandible as a good model for the evaluation of healing in a grafted defect. More recently, the sheep mandible model has been used to examine healing in extraction sockets (Lander, 2016; Liu et al., 2016) furcation defects (Baharuddin et al., 2015; Danesh-Meyer et al., 1997; Mohammed et al., 1998; Pack, 1997; Whelan et al., 1997), maxillary sinus defects (Smith et al., 2018) and dental implant related research (Duncan, 2005).

4.4.1 Healing Timelines for Extraction Sockets in Sheep

In animals, the location of a bone determines composition and turnover (Aerssens et al., 1998). Liebshner (2004) reported that trabecular bone from sheep vertebrae is much more dense than vertebral bone found in humans. Duncan (2005) reported the healing rate in the mandible was slower compared to the limbs in sheep over a similar healing period with sheep healing faster in comparison to humans (Duncan, 2005). It has been suggested that the healing time-points of 4, 8 and 16 weeks in sheep mandible correspond to 5, 11 and 21 weeks of healing in humans (Duncan, 2005).

The final healing time point of 16 weeks was chosen for the present study, as this in line with the healing sequence of human extraction sockets. Healing in the present study at 8 weeks was characterised by rapid changes in soft and hard tissues and this been also reported in previous studies at the same time point in the dog tooth extraction model, characterised by rapid osteoclastic activity resulting in dimensional changes (Araujo & Lindhe, 2005; Pagni et al., 2012). In our study, complete soft tissue healing was observed at 16 weeks with presence of lamellar bone and formation of woven bone. Studies in human extraction sockets between 12 and 24 weeks report the presence of lamellar bone, bone marrow, blood vessels, adipocytes, mesenchymal stem cells and inflammatory cells (Trombelli et al., 2008), with 60-65% of tissue volume present at 16 weeks of healing (Lindhe et al., 2012). Early implant placements are done at 4-8 weeks or 12-16 weeks post-extraction (Hämmerle & Jung, 2003).

4.4.2 Effects of Full Thickness Flap Elevation on Socket Healing

In our study, full thickness flaps were elevated prior to the extractions and primary closure was achieved for both non-grafted and grafted sockets. There is conflicting evidence regarding full thickness flap elevation and flapless approaches for ridge preservation. Flapless extraction can reduce additional bone loss (Wood et al., 1972). Furthermore, in dogs it has been reported that elevation of mucoperiosteal flaps led to reduced blood supply (Araujo & Lindhe, 2005) which can lead to osteocyte death and

eventually cause necrosis of the walls of the tooth socket (Schenk et al., 1994). Reduced bone resorption after 4 to 8 weeks healing following flapless extraction, both with and without ridge preservation, has been reported (Fickl et al., 2008). However, ridge preservation approaches did minimise bone resorption. Similar findings were reported by others (Blanco et al., 2008).

Research suggests that the initial differences between flapped and flapless extractions disappear after six months of healing (Araujo et al., 2009). Barone and colleagues (2013) carried out a randomised controlled trial study to study flapped and flapless approaches. Histological and histomorphometric assessments after 12 weeks of healing showed no differences between these approaches for alveolar ridge preservation. It has also been reported that secondary soft tissue closure and membrane exposure does not have any impact on the quality of bone regeneration (Barone et al., 2013).

In summary, there is evidence that differences are present during early healing between flapped and flapless extractions which disappear in the long-term and there is no general consensus regarding the preference of one technique over the other. In our study a flapped approach was used to facilitate extraction of teeth using minimal trauma without bone removal and tooth sectioning. In terms of level of difficulty for extraction of teeth in humans and sheep, tooth extractions in sheep are very laborious and technique sensitive. Using a flapped approach was also a suitable approach in sheep as they are ruminants with continuous mastication and their diets can be fibrous, therefore, primary closure was indicated to help stabilise the bone graft and membrane in the socket.

4.4.3 Use of Multiple Extraction Sites

In the present study, three consecutive premolars were extracted bilaterally creating an edentulous region. This is not usual standard practice in the clinical setting. The present study required more than one socket for control and test sites and having both in one animal made direct comparison possible. The literature regarding multiple extractions and its impact on healing is scarce. Al-Askar and colleagues (2013) assessed the dimensional changes comparing single versus multiple consecutive extractions in beagle

dogs. Microcomputed tomography assessments after 16 weeks of spontaneous healing extraction sockets showed more dimensional changes in sites with multiple extractions, especially when three teeth were extracted (Al-Askar et al., 2013). However, in a similar study in dogs, Al-Hamoudi and colleagues (2015) evaluated dimensional changes using microcomputed tomography in single versus multiple extraction sites that were grafted using xenograft and collagen membrane and found the dimensional changes for the single and multiple extractions sites were similar (Al-Hamoudi et al., 2015).

4.4.4 Animal Heterogeneity

The animals used in this study were commercially sourced and were not bred specifically for research purposes. The present study used a larger number of animals than previously used in other studies, it is therefore necessary to consider this when healing is compared between control and test groups. Animal heterogeneity is in fact advantageous when extrapolating *in vivo* results to clinical settings where individual variations in bone quality, bone remodelling and wound healing also exist (Jepsen et al., 2008).

4.5 Healing in the Animal Model

Sockets left to heal naturally or in the presence of Bio-Oss® and Bio-Gide® were both associated with soft tissue healing by 4 weeks followed by the formation of woven bone which started from the bottom of the defect and extended towards the lateral borders. A number of other studies have shown similar results in dogs (Araujo & Lindhe, 2005; Cardaropoli et al., 2003), monkeys (Scala et al., 2014) and the classic human study (Amler, 1969).

The sheep model has been used for many years to examine tooth extraction wound healing. Harrison described that bone formation can start from early as six days in sheep post-extraction (Harrison, 1943). Socket extraction in sheep model and ridge preservation has been previously reported in other studies (Lander, 2016; Liu et al., 2016). However, only the study by Liu and colleagues (2016) compared spontaneous healing with sockets grafted with xenograft or synthetic bone and collagen membrane using histomorphometry over 16 weeks of healing. Spontaneous healing was reported at 8 weeks, with the presence of immature woven bone and loose trabecular bone (Liu et al., 2016) which was similar to the present study. At 16 weeks, thick trabecular bone extending into the defect from both lingual and buccal aspects was present. For sockets grafted with synthetic bone, striations of trabecular bone from the buccal and lingual aspects extending into the centre of the sockets were reported (Liu et al., 2016). For sockets grafted with xenograft, particles of the biomaterials were in close proximity with woven and lamellar bone after 12 to 16 weeks of healing. However, in our study, minimal to no xenograft particles were observed in the defects from as early as four weeks of healing.

In a dog model, non-grafted extraction sockets have been observed and compared with sockets grafted with Bio-Oss® collagen, with more woven bone in the non-grafted sockets at two weeks compared to grafted sockets (Araujo & Lindhe, 2009). A direct comparison cannot be made as our earliest time point was four weeks. The authors suggested that the grafting material actually delayed healing in the early phases. Similar findings were have been reported by other researchers (Araujo & Lindhe, 2005; Cardaropoli et al., 2003, 2005). Cardaropoli and colleagues (2005) highlighted that after

tooth extraction, the residual periodontal ligament (PDL) in the apical region of the defect can retain some vitality and migrate into the provisional matrix during early healing. In our study, the socket sizes were standardised using implant burs, which would have removed any remaining PDL and consequently may have affected healing.

4.5.1 Behaviour of Grafting Materials in Alveolar Sockets in Sheep

Histological assessments of the grafted sockets in our study found that little grafting material remained in the sockets over time. Histological evidence from studies where biomaterials have been placed in healing sites have shown that they can cause a foreign body reaction that activates osteoclastic cells, resulting in the removal of the grafting material (Rasperini et al., 2010; Sisti et al., 2012). This could explain why limited amounts of grafting materials were observed in the sockets. Increased activity of osteoclasts in an attempt to remove the grafting materials from the socket has been translated to delayed healing (Canullo et al., 2016).

A split-mouth study in dogs compared spontaneous healing to a socket grafted with Bio-Oss® collagen (Araujo et al., 2008). At three months of healing, new mineralised bone made up of woven bone, parallel-fibered bone, lamella bone and graft particles was present over the defect entrance, demonstrating that the xenograft did not prevent bone remodelling but promoted formation of new bone in extraction defects despite little grafting material being present (Araujo et al., 2008). This finding is consistent with our study, however Araujo and colleagues used Bio-Oss® collagen, compared to the Bio-Oss® particulate graft that we used in our study.

The short- and long-term effects of grafting extraction sockets with xenografts (Bio-Oss® collagen) have been evaluated in a series of dog model studies (Araujo et al., 2009; Araujo & Lindhe, 2009). At two weeks of healing, there were no xenograft particles present in the apical portion of the defect with minimal amounts of woven bone present apically and laterally. At six months of healing, only about 12% of the xenograft was present in the socket and 80% of both non-grafted and grafted sockets were filled with mineralised bone. These findings further substantiate previous findings by Araújo and

colleagues that xenografts do not enhance bone formation but acts as a scaffold during the healing process. This scaffolding role of biomaterials like Bio-Oss® does however reduce the dimensional changes that occur during healing of extraction sockets.

One research group has reported large numbers of base multicellular units (BMUs) in grafted sockets compared to non-grafted sockets in a dog model (Buckwalter et al., 1995). BMUs were present at sites where active modelling/remodelling takes place, suggesting that xenografts may enhance the modelling/remodelling process. The authors also hypothesised that grafting material was present in the coronal aspect of the sockets, which was almost outside the actual defect, which could suggest that Bio-Oss® particles tend to get dislodged as they get replaced with new bone. Similar observations were made in our study, where the particles of Bio-Oss® appeared to be "pushed" out of the defect as healing and new bone formation progressed. This could also be explained by the reduced dimensional changes that take place in the grafted sockets during healing.

4.6 Immunohistochemistry – Discussion of Methodology

4.6.1 Decalcification and Paraffin Embedding

Histological assessment of mineralised tissue requires decalcification for removal of calcium ions/salts prior to paraffin embedding and staining. The process of decalcification involves submerging tissue specimens in acids or chelating solutions (Bandcroft & Gamble, 2002; Rolls et al., 2008). The properties of decalcifying solutions can be affected by concentration, pH and temperature. Duration of exposure and rate of penetration also plays an important role in decalcification (Savi et al., 2017).

In our study, alveolar sockets were decalcified using 10% EDTA. According to Savi and colleagues (2017) 10% EDTA at room temperature is the gold standard that gives optimal cellular and structural morphology. The decalcification time using 10% EDTA can be shortened by increasing the temperature of EDTA from room temperature to 37°C, however, this has adverse effects on soft tissue morphology. Decalcification with 5% nitric acid and 10% formic acids has been shown to be faster than EDTA, however these decalcifying agents caused adverse cellular and structural effects (Savi et al., 2017). Therefore, even though the decalcification process for mineralised tissue takes longer with 10% EDTA, the cellular and structural morphology is preserved and for this reason the present study used EDTA as a decalcification agent.

The time taken for complete decalcification of the sheep alveolar sockets ranged from 112 to 287 days. The sockets from the later time points (8 and 16 weeks) took longer to decalcify due to the presence of more woven bone as part of the healing process. Apart from the amount of woven bone, another factor that may have contributed to the variation was the different sizes of the alveolar sockets. The size of premolar sockets in sheep increases from anterior to posterior, therefore, third premolar sockets were larger than the first and second premolars. It has been suggested that the duration of decalcification process can be shortened in larger animals such as dogs, sheep and horses by cutting the specimens into smaller sequential sections (Schmitz et al., 2010). This strategy was also employed in the present study, where larger sockets were sectioned to enhance the decalcification process.

As this is the first study to carry out an immunohistochemical assessment of intact alveolar sockets harvested from sheep it was unknown how long this decalcification step would take. With this information now at hand a protocol is available for future research projects.

4.6.2 Tissue Section Adherence and Antigen Retrieval Techniques

Due to the presence of lamellar bone surrounding the alveolar socket, it was difficult to get the tissue sections to adhere to the slides. Tissue adherence to microscopic slides is based on close contact between the two surfaces. This close contact creates very weak bonds such as van der Waals forces or hydrogen bonds (Kiernan, 1999). However, these bonds are not sufficient to hold the tissues in place during the IHC process. Gambella and colleagues reported that thicker sections (7 μm) and aged slides (when packets were opened for longer than a week) could have negatively affected tissue adherence (Gambella et al., 2017). In terms of the brand of slides, Superfrost® slides was reported to lessen tissue detachment compared to HistoBond® (Gambella et al., 2017). Formalin fixation of 48 hours compared to 24 hours reduced tissue detachment significantly. Overall, tissue fixation in formalin for 24 hours for small samples to 48 hours for larger samples is standard practice and has shown to result in less tissue detachment (Gambella et al., 2017).

Heat induced epitope retrieval (HIER) and proteolytic enzyme-induced epitope retrieval (PIER) protocols are often used in IHC protocols for formalin-fixed and paraffin embedded tissues. Suurmeijer and Boon (1993) described the mechanisms of antigen retrieval, that included breaking down cross-linking between epitopes of unrelated proteins formed during formalin fixation; removal of diffusible blocking proteins; precipitation of proteins and hydration of tissue sections to enhance antibody diffusion and availability of epitopes (Suurmeijer & Boon, 1993).

The heat antigen retrieval method used for the anti-RANKL and anti-RANK antibodies made tissue adherence challenging. Therefore, various heat retrieval temperatures and duration were trialled to find the optimal temperature with the least tissue detachment. Ino (2003), reported that the optimal temperature for antigen heat retrieval to be above

90°C and the optimal retrieval solutions were a combination of distilled water and 10 mM sodium citrate, pH 6.0 (Ino, 2003).

A heat antigen retrieval method was not required for OPG as there were minimal concerns in relation to tissue detachment when slides were pre-treated with 0.1% trypsin in 0.1% $CaCl_2$ (pH 7.8) at RT for 10 mins.

In summary, tissue detachment is a common problem in IHC protocols and is dependent on various factors, some of that can be addressed such as tissue and slide conditions. Determining the optimum antigen retrieval protocol is an essential step in the success of the IHC procedure and needs to be tailored according to the tissue type and selected antibodies.

4.6.3 Antibody Selection and Optimisation

Antibodies used in previous studies can be divided into three categories; well-known antibodies associated with high quality evidence; well-known antibodies used in different species or unverified tissues and unknown antibody with scarce and unverified evidence (O'Hurley et al., 2014). It has been suggested that antibodies used in previous studies should be considered first (Acharya et al., 2017; Kim et al., 2016).

The RANKL and RANK primary antibodies that were initially selected were goat anti-mouse, however, during trials it was observed that there was a lot of non-specific staining which made it difficult to differentiate test slides from negative controls. A possible reason for this could have been species cross-reactivity between sheep and goat, as they share similar conserved and quaternary structures. The antigen binding site of an antibody identifies the immunoglobulins in one species, which is similar to the epitope on the immunoglobulins from another species. A review paper by O'Hurley, et al. (2014) highlighted that cross-reactivity can be avoided by ensuring that primary/ secondary antibodies are not raised in the same species. According to cellular genetic evidence, sheep and goat share a common ancestor, *Rupicarids* and goats have a similar karyotype to the ancestral form (Li et al., 2004), which could have been a possible reason for the cross-reactivity.

The second set of the primary antibodies for RANKL and RANK were rabbit anti-mouse and rabbit anti-human respectively and these were found to give lower background staining. As highlighted by Archarya and colleagues (2017), the sequences were aligned on NCBI thus confirming the correct protein was being detected.

Negative controls are an integral aspect of IHC protocol as antibody or non-specific binding and lack of secondary antibody specificity can lead to false-positive staining (Torlakovic et al., 2014). In summary, we have now developed detailed immunohistology protocols for the detection of RANK, RANKL and OPG in sheep alveolar sockets containing high amounts of lamellar bone.

4.7 Gene Analysis – Discussion of Methodology

4.7.1 Tissue Homogenisation

The present study is first to use regenerating tissue collected from a sheep tooth extraction socket. Studies that have used bone tissue from extraction sockets in humans have used homogenisation with a Bullet blender (Next Advance, Inc.) (Tan et al., 2019) or sonication with a blade (Canciani, et al., 2017). We found the Precellys® Evolution homogeniser with a cooling attachment (Cryolys) to be suitable for the preparation of a homogenous powder from the bony tissue sample with the full volume of homogenate recovered easily for RNA extraction. A high quality and quantity of RNA was then extracted.

4.7.2 Selection of Reference Genes

The quantification of mRNA expression is influenced by type of cell, RNA recovery and integrity, and cDNA synthesis efficiency (Vandesompele et al., 2002). To overcome the effect of any of these factors on the expression of a gene of interest (GOI), reference or housekeeping genes which are unaffected by the experimental conditions are used to normalise the GOI expression level.

Only two studies have evaluated bone-related reference genes in sheep (Jiang et al., 2015; Schulze et al., 2017). In the present study, B2M, ACTB, GAPDH, RPL19 and YWHAZ were evaluated for their suitability as reference genes. B2M was found to be consistently expressed at a high level and was judged suitable to be used as a reference gene. Schulze and colleagues (2017) also used B2M along with RPL19 and YWHAZ for quantification of gene expression in ovine bone.

4.8 Study Limitations

Sample collection was carried out post euthanasia. The mandible was disarticulated and resected before x-ray images were taken. Each socket was identified by superimposing the radiograph over the mandible and aligned with landmarks around the teeth and bone. The premolar socket outlines were marked on the mandible and sectioned using a saw. A similar method of socket sectioning was used by Lander and colleagues (2016). Some angulation and distortions did arise from the radiographic step which made matching the socket difficult however, this method proved to be fairly accurate in producing individual tooth sockets for histological assessments.

For future research, the use of radiographic markers such as amalgam restorations or pins to demarcate individual sockets should be considered. However, because sheep are ruminants, these radiographic markers should be stable enough to last the duration of the study without getting dislodged or lost. Another method to ensure accuracy in sectioning individual sockets would be to take impressions of the teeth prior to extractions and construct stents for individual sheep. However, this method will increase operative time and add additional cost, especially with large sample sizes.

Tissue samples for mRNA analysis were collected from the third premolar site which was mesial to the first molar. This site was chosen for ease of identification using the first molar as a reference point. A similar method of tissue harvesting was previously reported by Caniciani and colleagues (2017), where periapical radiographs were used to identify the centre of the third premolar socket and a trephine used to collect tissues samples from human non-grafted and grafted alveolar sockets. Guidance stents were not used in this study. Apart from the use of stents, three-dimensional computed tomography (CT) or cone-beam CT (CBCT) could have provided valuable additional information, unfortunately this was not available at the time.

4.9 Conclusions and Clinical Significance

4.9.1 Conclusions

In sheep tooth sockets grafted with Bio-Oss® and Bio-Gide®, histological examination suggested that more woven bone appeared to be present, likely due to the lower level of RANK expression resulting in decreased osteoclastic activity. There was, however, no statistically significant difference in the expression of the key markers of osteogenesis, RANKL and OPG between empty and grafted sockets at any time over the 16-week period. There were also no differences in the expression of the transcription factors of osteoblast differentiation MSX2 or the ECM markers Col1A1 or TIMP3. Even though Bio-Oss® is not osteogenic, its use for alveolar ridge preservation should be continued due to its osteoinductive and scaffolding properties.

4.9.2 Clinical Significance

Soft and hard tissue remodelling is a natural response in natural healing of extraction sockets. The remodelling process can lead to 40-60% dimensional changes in the remaining alveolar ridge (MacBeth et al., 2017). To minimise alveolar bone loss and maximise bone formation in the socket, alveolar ridge preservation techniques are used. These include minimally traumatic extractions and grafting the socket with bone and/or bone substitutes with or without barrier membranes. Currently, no treatment protocol or biomaterial is more superior than the others (Atieh et al., 2015; MacBeth et al., 2017). Dental implants are therefore, placed simultaneously with grafting or after a period of healing (delayed-type placement). The quality and quantity of bone for achieving primary stability determines the timing of implant placements.

Even though studies have demonstrated that autogenous bone or biomaterials do not completely prevent bone resorption (Araujo & Lindhe, 2011; Araujo et al., 2015); ARP procedures do maintain the horizontal and vertical dimensions compared to non-grafted sites (Apostolopoulos & Darby, 2017). This allows placement of dental implants without additional grafting at ARP sites (Cardaropoli et al., 2014). Implant survival rates in non-grafted sites range from 97-100% (Albrektsson et al., 1986; Bergenblock, Andersson et al., 2012; Buser et al., 1998; Leonhardt et al., 2002; Lindquist, Carlsson, & Jemt, 1997);

and implants placed in ridge preserved site have reported survival rates of 93-100% (Norton & Wilson, 2002; Patel et al., 2013). Patel and colleagues (2013) reported that the success rate of implants placed in ridge preserved sites with Bio-Oss® to be 83% at 12 months of loading. In a more recent study, the success rate of implants placed in ridge preserved site was 51% compared to 58% in non-grafted sites at 31 ± 24 months (Apostolopoulos & Darby, 2017). The differences in success rates reported in various studies can be attributed to the use of different criteria. These studies demonstrate that ridge preserved sites are comparable to naturally healing sockets in terms survival and success of dental implants.

The present study has demonstrated at a molecular level that the RANK/ RANKL/ OPG axis plays a key role in healing and remodelling of extraction sockets. Within its limitations, the present study has shown that healing at a molecular level, as shown by the expression of key osteogenic markers and regulators of osteoclast differentiation, was comparable in sockets grafted with Bio-Oss® and non-grafted sockets in a sheep model.

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Appendices

Appendix I: Sample Processing

1. Randomization (Latin Square) for Treatment Allocation

Surgery No.	Sheep No.	Group	R P1	R P2	R P3
1	597	4- week	A	B	C
2	437	4-week	B	C	A
3	432	4-week	C	A	B
4	586	4-week	A	B	C
5	590	4-week	B	C	A
6	441	4-week	C	A	B
7	583	4-week	A	B	C
8	593	4-week	B	C	A
9	428	4-week	C	A	B
10	595	4-week	A	B	C
11	440	8-week	A	B	C
12	594	8-week	B	C	A
13	589	8-week	C	A	B
14	434	8-week	A	B	C
15	591	8-week	B	C	A
16	598	8-week	C	A	B
17	426	8-week	A	B	C
18	439	8-week	B	C	A
19	438	8-week	C	A	B
20	450	8-week	A	B	C
21	430	16-week	A	B	C
22	436	16-week	B	C	A
23	599	16-week	C	A	B
24	584	16-week	A	B	C
25	587	16-week	B	C	A
26	585	16-week	C	A	B
27	442	16-week	A	B	C
28	592	16-week	B	C	A
29	427	16-week	C	A	B
30	425	16-week	A	B	C

2. Specimen Coding for RNA Extraction and Gene Analysis

Unique Number	Sheep Number	Group	R P3
1	597	4-week	C
2	432	4-week	B
3	586	4-week	C
4	441	4-week	B
5	583	4-week	C
6	428	4-week	B
7	595	4-week	C
8	440	8-week	C
9	589	8-week	B
10	434	8-week	C
11	598	8-week	B
12	426	8-week	C
13	438	8-week	B
14	450	8-week	C
15	430	16-week	C
16	599	16-week	B
17	584	16-week	C
18	585	16-week	B
19	442	16-week	C
20	427	16-week	B
21	425	16-week	C

RP3: lower right third premolar

Appendix II: Buffer Preparation

1. 10% EDTA (ethylenediamenetetraacetic acid)

Materials:

- EDTA powder (Titriplex® III), Cat. No. 1.084818.1000 (Merck)
- Sodium hydroxide, Cat. No. 1.06498.0500 (Merck)
- Double distilled water (dd H_2O)
- Milli-Q water (MQ H_2O)

Equipment:

- Beaker
- Flask
- Magentic stirrer
- Magnetic mixer
- pH meter
- Digital balance
- Autoclave

Procedure (for making 750 mL of 10% EDTA solution):

- Wash all glass wear and magentic stirrer with distilled water
- Weigh out 75 grams of EDTA powder into a large beaker
- Add 500 mL of double distilled water and stir gently using a magentic stirrer unitl well dispered.
- Wash and calibrate a pH probe with MQ H_2O
- Adjust the pH to 7.4 using approximately 40 pellets of sodium hydroxide
- Add dd H_2O to bring the volume to ~700 mL
- When the solution is clear the pH should be checked again.
- Transfer the solution to a measuring cylinder and bring the volume to 750 mL with double distilled water
- Transferred to a clean labelled bottle
- Ensure the bottle top is half-loose and autoclave indictor tape placed on top of the lid
- Autoclave at 121°C for 40 minutes

- Close the lid when removed from the autoclave.

2. Ammonium Oxalate Test for decalcification method

Materials:

- Concentrated Hydrochloric Acid (cHCl) (Merck, Cat #: 100317.2500, Germany)
- Di-Ammonium Oxalate Monohydrate (Merck, Cat #: 1.01190.1000, Germany)
- Phosphate Buffered Saline (PBS) (Gibco™, cat #: SALB101)

Procedure:

- Remove EDTA decalcification solution (5ml) from the specimen pot
- Add concentrated 1M HCl until pH in range 3.2 – 3.6 (test with litmus paper)
- Add 5 mL ammonium oxalate solution
- Incubate at room temperature for 30mins
- A clear solution indicates decalcification is complete, transfer to PBS for paraffin embedding. If precipitate detected, return to pot and continue decalcification as per protocol

3. Antigen Retrieval Solution (Heat) 0.1M Tri-sodium Citrate for IHC

Materials:

Tri-sodium Citrate dihydrate (ACS, ISO, Reag, Ph Eur), (Merck, Cat. No. 1.06448.0500)

MW = 294.10 g/mol

- Distilled water (d H_2O)
- 10 M hydrochloric acid (HCl)

Equipment:

- Beaker (1 L)
- Measuring cylinder (1 L)
- Magentic stirrer
- pH meter
- Digital balance
- Filter sterilizer (Nalgene®, Cat #: 565-0020)

- Schott bottle

Procedure for 1 L

- Measure 29.4 g of tri-sodium citrate power into a 1 L beaker
- Add 800 mL of distilled water
- Mix well using a magnetic stirrer
- Wash and calibrate a pH probe with MQ H_2O
- Adjust the pH to 6.0 using approximately 30 drops of 10 M HCl
- Add d H_2O to bring the volume to ~900 mL
- Once the pH reachs 6.0 the solution should be transferred into a measuring cylinder
- Bring volume to 1 L with distilled water
- Sterilise the solution by filtration into labelled, autoclaved schott bottle.
- Store at room temperature

4. Antigen Retrieval Pre-treatment Reagent 0.1% Trypsin in 0.1% $CaCl_2$ for IHC

Materials:

- Calcium chloride dihydrate (Merck, Emsure®, cat #: 1.02382.0250)
- Trypsin (Sigma-Aldrich, cat # T7409)
- 0.1 N NaOH
- Distilled H_2O

Equipment:

- Beaker
- Flask (250 mL)
- Magentic stirrer and magnetic bar
- pH meter
- Digital balance
- Filter sterilizer: 0.22 µm (Nalgene®, Cat # 565-0020, Lot # 1033169)
- Storage bottle

Procedure:

Part A: Prepare 0.1% $CaCl_2$ pH 7.8

- Weigh 100 mg of $CaCl_2$ dihydrate in a beaker
- Add 100 mL of dH_2O and mix using a magentic stirrer
- Bring the pH to 7.8 by adding 0.1 N NaOH
- Filter sterilize and store at room temperature

Part B: Prepare 0.1% trypsin

- To 11 mg of trypsin add to 11 mL of 0.1% $CaCl_2$ solution
- Aliquot 500 µl into 1.5 mL tubes
- Label and store at -20^{0}C

5. Wash Buffer (0.1% Tween-20 and 0.5% milk powder in PBS) for IHC

Materials:

- PBS (Gibco™, Invitrogen, cat #: SALB010)
- Tween-20 (BIO-RAD, cat #:170-6531)
- 0.5% milk powder (Pams)
- 1 mL pipette tip

Equipment:

- Magnetic stirrer and bar
- Digital scale
- Scott bottle (500 mL)

Procedure:

- To 320 mL of PBS, add 1.6 g of non-fat milk powder and 320 µL Tween-20 (cut end of 1 mL pipette tip)
- Stir until clear (in magnetic stirrer with magnetic bar)
- Store in 4°C room before use

Appendix III: Immunohistochemistry

1. Tissue Processing (Paraffin Embedding)

Automated Thermo Scientific™ Excelsior™ ES, Germany

Program: Routine Overnight

Step No.	Reagent	Hold/ Use Temperature	Time (min)
1.	Formalin	Ambient	30
2.	Formalin	Ambient	30
3.	Alcohol	Ambient	60
4.	Alcohol	Ambient	60
5.	Alcohol	Ambient	60
6.	Alcohol	Ambient	60
7.	Alcohol	Ambient	60
8.	Alcohol	Ambient	90
9.	Xylene	Ambient	60
10.	Xylene	Ambient	60
11.	Xylene	Ambient	90
12.	Wax	62°C	60
13.	Wax	62°C	60
14.	Wax	62°C	30

2. Slide Tissue Adherence Study

Test 1: Tissue Adherence on Different Slides

Representative study tissue sections (4 µm) from sample (583 RP3 A) were incubated at 60^0C overnight on three different brands of slides:

Slide #	Slide Type	Code
1	Uber (currently used in the laboratory)	X1
2	Uber	X2
3	Adhesive Trajan (Trajan Scientific and Medical, Lot # 806052N-SP3A)	Y1
4	Adhesive Trajan (Trajan Scientific and Medical, Lot # 806052N-SP3A)	Y2
5	Crest Adhesive (Matsunami Glass Ltd, Lot # 803158R-SP3A)	Z1
6	Crest Adhesive (Matsunami Glass Ltd, Lot # 803158R-SP3A)	Z2

Test 2:

The tissues were cut at a thickness of 4µm from sample (583 RP3 B). Frosted slides were used. Menzel-Glaser, Superfrost Ultra Plus® (Thermo Scientific, Cat # J3800AMNZ) were used at different temperatures.

Slide #	Slide Type	Temperature	Code
1	Superfrost Ultra Plus®	Heat (60°C)	A/ heat
2	Superfrost Ultra Plus®	Heat (60°C)	B/ heat
3	Superfrost Ultra Plus®	Room temperature	A/ RT
4	Superfrost Ultra Plus®	Room temperature	B/ RT

The following method was used for the above test

1. Clearly label slides with HB pencil.
2. De-wax and rehydrate tissue sections *(Xylene (HSNO, Batch #: 1701236660, Cat #: AJA2342-5L) and graded Alcohol)*
    i. De-Wax with Xylene: (held in Coplin jars with lids, labelled)
        a. Xylene 1: 5 minutes
        b. Xlylene 2: 5 minutes
        c. Xlylene 3: 10 minutes

ii. Rehydrate with Alcohol (different concentrations, held in Coplin jars with lids, labelled)
    a. Alcohol 1: 100% 5 minutes
    b. Alcohol 2: 90% 5 minutes
    c. Alcohol 3: 70% 5 minutes
    d. Alcohol 4: 50% 5 minutes
iii. Wash with dH_2O - 2 times for 2 minutes each

3. Antigen Retrieval *(Tri-sodium citrate 0.1 M)*

- Heat retrieval method chosen:
    - Add 500 mL dH_2O to the Decloaking Chamber NxGen metal water chamber
    - Add 220 mL dH_2O to the two outside metal slide canisters (left and right)
    - Except the one with the slides inside – to which you should add 220 mL antigen retrieval solution (10x Tri Sodium Citrate 0.1 M → pH 6.0; Dilute 1:9 to 0.01 M (working concentration) before use by adding 22 mL of 10 x concentrate to 198 mL of dH_2O
    - Choose the program, which is 110°C for 10 mins. After finishing, wait the machine cool down to 90°C and take out of the metal slide canister
    - Wash the slides 2 times for 5 minutes in a 1 L beaker with around 450 mL PBS (can be shaken).
    - The citrate buffer could only be used once and be poured off through the sink.

End Point Outcomes: To observe which slide type has best tissue adherence after heat retrieval.

Conclusion: Superfrost slides showed the best tissue adherence for the tissue samples.

3. Sequence of Fluids used on Automated Staining Machine (Shandon Varistain 24-3)

Sequence No.	Reagent	Time
1.	Xylene	1 minute
2.	Xylene	30 seconds
3.	Xylene	30 seconds
4.	Absolute Alcohol	30 seconds
5.	Absolute Alcohol	30 seconds
6.	Absolute Alcohol	30 seconds
7.	Absolute Alcohol	30 seconds
8.	Absolute Alcohol	30 seconds
9.	Absolute Alcohol	30 seconds
10.	Absolute Alcohol	30 seconds
11.	Absolute Alcohol	30 seconds
12.	Running water	30 seconds
13.	Gill's No. 2 Haematoxylin	1 minute
14.	Running water	30 seconds
15.	Scott's tap water	30 seconds
16.	Running water	30 seconds
17.	Eosin	1 minute 30 seconds
18.	Running water	30 seconds
19.	Absolute Alcohol	30 seconds
20.	Absolute Alcohol	30 seconds
21.	Xylene	30 seconds
22.	Xylene	30 seconds
23.	Xylene	30 seconds

4. Trichrome Staining Protocol

Materials:

- Trichrome staining kit (ab150686, Abcam)
- Absolute alcohol
- Xylene

All reagents were prepared just prior to use, with gently agitation, and equilibrated to room temperature.

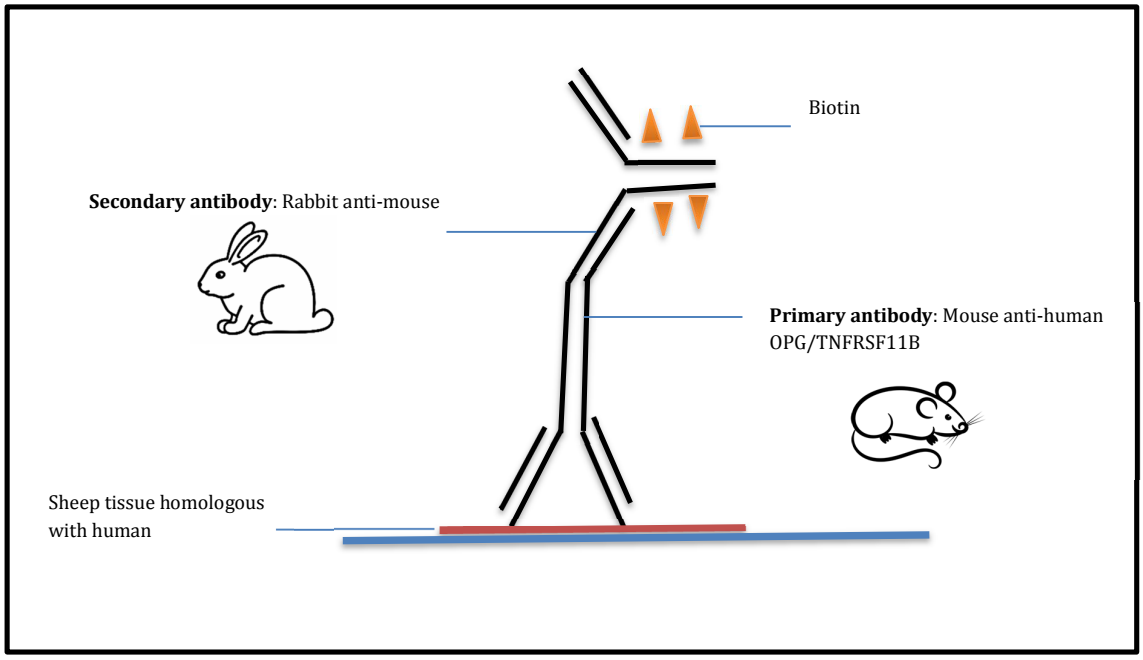
Procedure:

- All sections were deparaffinised and hydrated in dH_2O
- Bouin's Fluid was preheated in a water bath at 56-64°C in the fume hood
- Slides were placed in preheated Bouin's Fluid for 60 mins followed by a 10 mins cooling period
- Slides were rinsed in tap water until sections were completely clear, followed by one rinse in dH_2O
- Equal parts of Weigert's (A) and Weigert's (B) were mixed and slides were stained with working Weigert's Iron Hematoxylin for 5 mins. Slides were then rinsed in running tap water for 2 mins
- Biebrich Scarlet/ Acid Fuchsin solution was applied to slides for 15 mins and rinsed in dH_2O
- Slides were differentiated in Phosphomolybdic/ Phosphotungstic Acid solution for 10-15 mins or until collagen was not red. Slides were not rinsed.
- Aniline Blue Solution was applied to slides for 5-10 mins, and then rinsed in distilled water
- 1% Acetic Acid Solution was applied to slides for 3-5%
- Slides were then dehydrated very quickly in 2 changes of 95% Alcohol followed by 2 changes of Absolute Alcohol
- Slides were cleared in xylene and mounted in synthetic resin

5. Immunohistochemistry Protocols

A. OPG

Primary Ab	Isotype matched Control	Secondary Ab
Anti-OPG Mouse anti-human OPG/TNFRSF11B Cat #. NB100-56505 (98A1071), Novusbio Comes as 1.0 mg/mL (1000 µg/mL (needs to be diluted in PBS) Working stock 0.4 mg/mL will be made from 1.0mg/mL	Mouse IgG (sc2025), Stock: 0.4 mg/mL	Rabbit anti-mouse (Biotin) (ab5761), 0.5 mg/mL, Dilution range 1:1000-1:5000, Use at 1:400.
Blocking Serum: rabbit serum		



Day 1

De-waxing

Label slides and mark tissue sections with wax pen
Xylene: 1-3
Timings: 5 minutes 2x and 10 minutes 1x

Rehydration

Alcohol
100%, 90%, 70%, 50% - 5 minutes each
Distilled water - 2x for 2 minutes each

Pre-treatment

Use 0.1% trypsin in 0.1% $CaCl_2$ (pH 7.8) → 10 mins at RT. Add 150µl per slide
Wash with PBS 2x 5mins each

Blocking

20% Rabbit Serum (heat treated) + 1% BSA/ PBS (Blocking Serum (BS) = ~150 µl/ slide Store on ice. Incubate at room temperature for 30 minutes (humidified chamber). No washing after blocking
Total 20 slides Total volume of required: 3000µl = 150µl x 20 slides
RS volume required (600 µl) = 3000 x 0.2
Volume of **1% BSA/PBS required (2400 µl)** = Total volume (3000 µl) - Total volume of rabbit serum (600µl)

Immunohistochemical Staining

Ab diluent: **5% rabbit serum** + **1% BSA/ PBS**
Ab diluent required = **3000** µl (150 µl 5% rabbit serum + **2850µl 1% BSA/PBS**) → store ice
Total volume of Ab diluent required = 3000 µl (150*20=3000)
Volume of rabbit serum required = Total volume of required (3000 µl) x 0.05
Volume of 1% BSA/PBS required = Total volume - Volume of 5% RS

Apply diluent **primary antibody**
5 µg/ml OPG (1: 200 dilution) →
3000 µl required = 15 µl Ab + **2985 µl of diluent**

3000µl (150*20)
Volume of ab = total required/ dilution
Volume of diluent required = total required - volume of ab

Add diluted 1° Ab on all **test** slides → 150µl/ slide

Incubate

Overnight at 4^0C with PBS

Day 2

Wash Buffer → Wash buffer (PBS for 45 mins) → shaking 3x changes (5, 20, 20mins)

Secondary Antibody → 150µl/ slide, calculate for total number of slides
(3000µl for 20 slides) →3000/400 (dilution factor) = 7.5 µl (secondary Ab) 1:400 dilution
3000 µl - 7.5 µl = **2992.5 µl of diluent**
Incubate for 1 hour in incubation box at RM

Wash with PBS for 5, 10, 10 mins → 3 changes

Endogenous Perioxidase Blocking → Incubate slides in 0.3% H_2O_2 in methanol (prepared by adding 1ml of 30% H_2O_2 in 100ml of methanol and cover with parafilm→ made fresh) → incubate for 10 mins
Wash with PBS 3x 5mins each

Advin-biotin-horseradish Peroxidase → Vectorstain Elite ABC used:
Mixture → solution A (**120** µl) + PBS (**3000** µl) + solution B (**120** µl) mix and wait for 30 mins
For 1000 µl of PBS → need 0.04*1000 (for every 1000) = solution A (same for solution B)
For 20 slides = 3000 µl required (3000*0.04=120 A&B)
Add 150 µl/ slide → incubate for 30 mins at RT in incubation box
Wash with PBS 3x → 5, 10, 10 mins

3,3' diaminobenidine (DAB) chromogen → Mixture →DAB solution B (**90** µl) + Buffer solution A (**2910** mL)
DAB solution B (~50 µl/ 1 drop) to 1000 µl buffer solution A
For 20 slides = 3000 µl →
90µl DAB solution B + 2910 µl buffer solution A
Add 150 µl/ slide → incubate for 3 mins (3 slides/ batch)
Discard DAB in separate bottle
Wash with dH_2O: 3 x 5 mins

Haematoxylin Stain →
a. Haematoxylin → 5 secs
b. Running tap water → until clear
c. Scott's water → 1 min
d. Running cold tap water → few secs
e. 50% alcohol → 2 mins
f. 90% alcohol → 2 mins
g. 100% alcohol → 2 mins
h. Xylene → 2 x 5 mins

Mounting → 2 drops of DepeX → coverslip →dry

Day 1

B. Mouse IgG

De-waxing

Label slides
Xylene: 1-3
Timings: 5 minutes 2x and 10 minutes 1x

Rehydration

Alcohol 100%, 90%, 70%, 50% - 5 minutes each
Distilled water - 2x for 2 minutes each
Mark sections with wax pen

Pre-treatment

Use 0.1% trypsin in 0.1% $CaCl_2$ (pH 7.8) → 10 mins at RT
Add 150µl per slide. Wash with PBS 2x 5mins each

Blocking

20% Rabbit Serum (heat treated) + 1% BSA/ PBS (Blocking Serum (BS) = ~150 µl/ slide Store on ice. Incubate at room temperature for 30 minutes (humidified chamber). No washing after blocking
Total 20 slides Total volume of required: 3000 µl = 150 µl x 20 slides
RS volume required (600 µl) = 3000 x 0.2
Volume of **1% BSA/PBS required (2400 µl)** = Total volume (3000 µl) - Total volume of rabbit serum (600 µl)

Immunohistochemical Staining

Ab diluent: **5% rabbit serum** + **1% BSA/ PBS**
Ab diluent required = **3000** µl (150 µl 5% rabbit serum + **2850 µl 1% BSA/PBS**) → store ice
Total volume of Ab diluent required = 3000 µl (150*20=3000)
Volume of rabbit serum required = Total volume of required (3000 µl) x 0.05
Volume of 1% BSA/PBS required = Total volume - Volume of 5% RS

Apply diluent **mouse IgG** (concentration = 400 µg/ ml)
5 µg/ml IgG (1: 80 dilution) → 400/5=80
3000 µl required = 37.5 µl IgG + **2962.5 µl of diluent**

3000µl (150*20)
Volume of IgG = total required/ dilution
Volume of diluent required = total required - volume of IgG

Add diluted 1° IgG on all **test** slides → 150 µl/ slide

Incubate

Overnight at 4^0C with PBS

Day 2

Wash Buffer → Wash buffer (PBS for 45 mins) → shaking 3x changes (5, 20, 20mins)

Secondary Antibody → 150µl/ slide, calculate for total number of slides
(3000µl for 20 slides) →3000/400 (dilution factor) = 7.5 µl (secondary Ab) 1:400 dilution
3000 µl - 7.5 µl = **2992.5 µl of diluent**
Incubate for 1 hour in incubation box at RM

Wash with PBS for 5, 10, 10 mins → 3 changes

Endogenous Perioxidase Blocking → Incubate slides in 0.3% H_2O_2 in methanol (prepared by adding 1 mL of 30% H_2O_2 in 100 mL of methanol and cover with parafilm→ made fresh) → incubate for 10 mins
Wash with PBS 3x 5mins each

Advin-biotin-horseradish Peroxidase → Vectorstain Elite ABC used:
Mixture → solution A (**120** µl) + PBS (**3000** µl) + solution B (**120** µl) mix and wait for 30 mins
For 1000µl of PBS → need 0.04*1000 (for every 1000) = solution A (same for solution B)
For 20 slides = 3000 µl required (3000*0.04=120 A&B)
Add 150 µl/ slide → incubate for 30 mins at RT in incubation box
Wash with PBS 3x → 5, 10, 10 mins

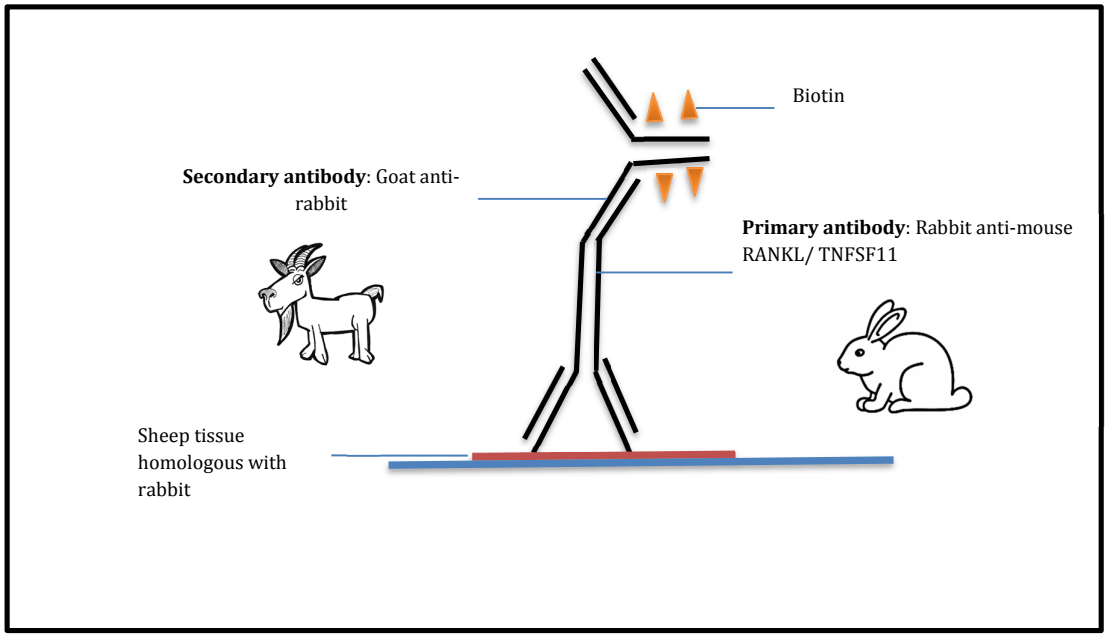
3,3' diaminobenidine (DAB) chromogen → Mixture →DAB solution B (**90** µl) + Buffer solution A (**2910** mL)
DAB solution B (~50 µl/ 1 drop) to 1000 µl buffer solution A
For 20 slides = 3000 µl →
90 µl DAB solution B + 2910 µl buffer solution A
Add 150 µl/ slide → incubate for 3 mins (3 slides/ batch)
Discard DAB in separate bottle
Wash with dH_2O: 3 x 5 mins

Haematoxylin Stain →
a. Haematoxylin → 5 secs
b. Running tap water → until clear
c. Scott's water → 1 min
d. Running cold tap water → few secs
e. 50% alcohol → 2 mins
f. 90% alcohol → 2 mins
g. 100% alcohol → 2 mins
h. Xylene → 2 x 5 mins

Mounting → 2 drops of DepeX → coverslip →dry

C. RANKL

Primary Ab	Isotype matched Control	Secondary Ab
Anti-RANKL Rabbit anti-mouse TNFSF11/RANKL Cat #: (ab216484), Abcam 1mg/mL Use at 1:200	Rabbit IgG (Lot #: Dako X0936), Stock: 1 mg/mL Use at 1 :200	Goat anti-rabbit (Biotin) (ab6012) 1 mg/mL, Dilution used at 1:200.
Blocking serum: Goat Serum **Antigen Retrieval:** Heat		



Day 1

De-waxing

Xylene: 1-3
Timings: 5 minutes 2x and 10 minutes 1x

Rehydration

Alcohol 100%, 90%, 70%, 50% - 5 minutes each
Distilled water 2x for 2 minutes each

Antigen Retrieval

80°C for 15 mins in Vector H-3300 (Tri-sodium citrate → dilute 1:9)
Working concentration for tri-sodium citrate = 22 mL of stock solution + 198 mL of dH_2O
Add 220ml of dH_2O in 2 outside containers, 500 mL dH_2O in the main pot
After heat retrieval → wash with PBS 2x 5mins
Mark sections with wax pen

Blocking

20% Goat Serum (heat treated) + 1% BSA/ PBS
(Blocking Serum (BS) = ~150 µl/ slide Store on ice.
Total for 20 slides
Incubate at room temperature for 30 minutes (humidified chamber)
Total volume of required: 3000 µl = 150 µl x 20 slides
Volume required (600 µl) = 3000 x 0.2
Volume of 1% BSA/PBS required (2400 µl) = Total volume (3000 µl) - Total volume of goat serum (600 µl)
Do not rinse off, wipe excess

Immunohistochemical Staining

Ab diluent → 5% goat serum + 1% BSA/ PBS
Mix in 1.5 mL eppendorf tube (account for day 1 + 2)
Ab diluent: 5% goat serum + 1% BSA/ PBS in 1.5ml eppendorf tube
Ab diluent required = **7000** µl (350 µl 5% goat serum + 6650 µl 1% BSA/PBS → store ice
Total volume of Ab diluent required = 70**00** µl (for day 1 and 2)
Volume of goat serum required = Total volume of required x 0.05
Ab diluent → 5% goat serum + 1% BSA/ PBS
Mix in 1.5ml eppendorf tube (account for day 1 + 2)

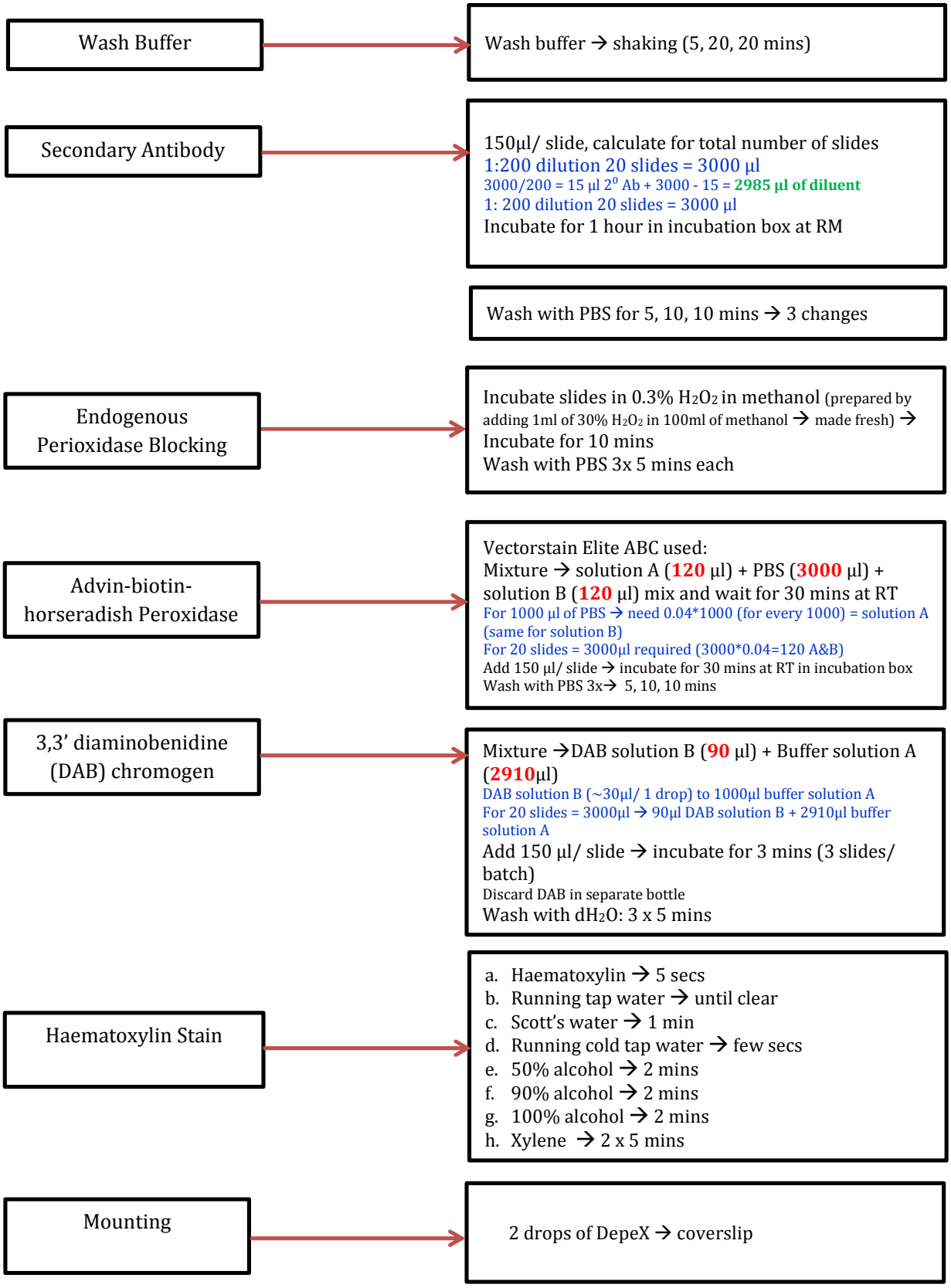
Apply diluent **primary antibody**
5 µg/ml RANKL: 3000 µl (1: 200 dilution) → 3000 µl required = 15 µl Ab + **2985 µl of diluent**
Volume of ab = total required/ dilution
3000/200 =15 µl of Ab + 2985 µl of diluent
Volume of diluent required = total required - volume of ab
Volume of ab = total required/ dilution

Add diluted 1° Ab on all **test** slides → 150 µl/ slide

Incubate

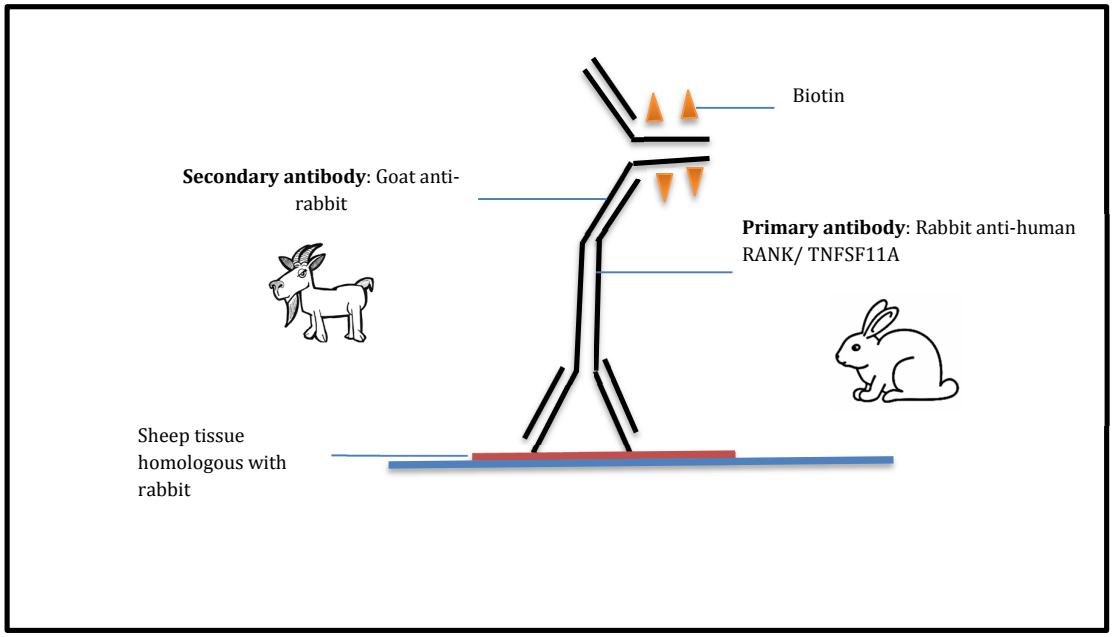
Overnight at 4°C with PBS

Day 2


Wash Buffer
Wash buffer → shaking (5, 20, 20 mins)
Secondary Antibody
150µl/ slide, calculate for total number of slides
1:200 dilution 20 slides = 3000 µl
3000/200 = 15 µl 2° Ab + 3000 - 15 = 2985 µl of diluent
1: 200 dilution 20 slides = 3000 µl
Incubate for 1 hour in incubation box at RM
Wash with PBS for 5, 10, 10 mins → 3 changes
Endogenous Perioxidase Blocking
Incubate slides in 0.3% H2O2 in methanol (prepared by adding 1ml of 30% H2O2 in 100ml of methanol → made fresh) →
Incubate for 10 mins
Wash with PBS 3x 5 mins each
Advin-biotin-horseradish Peroxidase
Vectorstain Elite ABC used:
Mixture → solution A (120 µl) + PBS (3000 µl) + solution B (120 µl) mix and wait for 30 mins at RT
For 1000 µl of PBS → need 0.04*1000 (for every 1000) = solution A (same for solution B)
For 20 slides = 3000µl required (3000*0.04=120 A&B)
Add 150 µl/ slide → incubate for 30 mins at RT in incubation box
Wash with PBS 3x→ 5, 10, 10 mins
3,3' diaminobenidine (DAB) chromogen
Mixture →DAB solution B (90 µl) + Buffer solution A (2910µl)
DAB solution B (~30µl/ 1 drop) to 1000µl buffer solution A
For 20 slides = 3000µl → 90µl DAB solution B + 2910µl buffer solution A
Add 150 µl/ slide → incubate for 3 mins (3 slides/ batch)
Discard DAB in separate bottle
Wash with dH2O: 3 x 5 mins
Haematoxylin Stain
a. Haematoxylin → 5 secs
b. Running tap water → until clear
c. Scott's water → 1 min
d. Running cold tap water → few secs
e. 50% alcohol → 2 mins
f. 90% alcohol → 2 mins
g. 100% alcohol → 2 mins
h. Xylene → 2 x 5 mins
Mounting
2 drops of DepeX → coverslip

D. RANK

Primary Ab	Isotype matched Control	Secondary Ab
Anti-RANK Rabbit anti-human TNFSF11A/RANK Cat #: (CD265), Invitrogen 0.5mg/mL Use at 1: 100	Rabbit IgG (Lot #: Dako X0936), Stock: 1 mg/mL Use at 1: 200	Goat anti-rabbit (Biotin) (ab6012) 1 mg/mL, Dilution used at 1:200.
Blocking serum: Goat Serum **Antigen Retrieval:** Heat		



Day 1

De-waxing

Xylene: 1-3
Timings: 5 minutes 2x and 10 minutes

Rehydration

Alcohol 100%, 90%, 70%, 50% - 5 minutes each
Distilled water 2x for 2 minutes each

Antigen Retrieval

80°C for 15 mins in Vector H-3300 (Tri-sodium citrate → dilute 1:9)
Working concentration for tri-sodium citrate = 22ml of stock solution + 198ml of dH_2O
Add 220ml of dH_2O in 2 outside containers, 500ml dH_2O in the main pot
After heat retrieval → wash with PBS 2x 5mins
Mark sections with wax pen

Blocking

20% Goat Serum (heat treated) + 1% BSA/ PBS
(Blocking Serum (BS) = ~150 µl/ slide Store on ice.
Total for 20 slides
Incubate at room temperature for 30 minutes (humidified chamber)
Total volume of required: 3000 µl = 150µl x 20 slides
Volume required (600 µl) = 3000 x 0.2
Volume of 1% BSA/PBS required (2400µl) = Total volume (3000 µl) - Total volume of goat serum (600 µl)
Do not rinse off, wipe excess

Immunohistochemical Staining

Ab diluent → 5% goat serum + 1% BSA/ PBS
Mix in 1.5ml eppendorf tube (account for day 1 + 2)
Ab diluent: 5% goat serum + 1% BSA/ PBS in 1.5ml eppendorf tube
Ab diluent required = **7000** µl (350 µl 5% goat serum + 6650 µl 1% BSA/PBS → store ice
Total volume of Ab diluent required = 70**00** µl (for day 1 and 2)
Volume of goat serum required = Total volume of required x 0.05

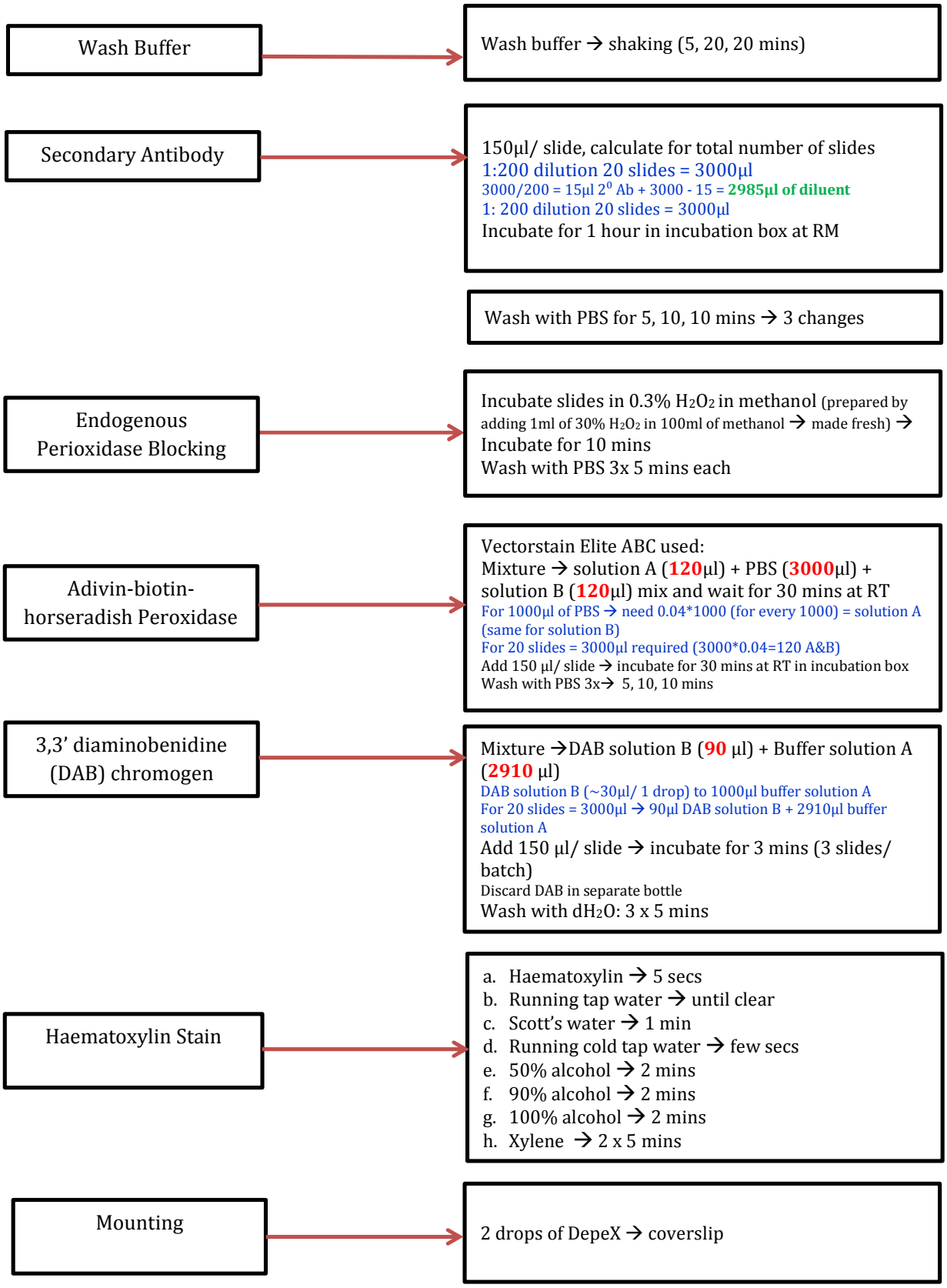
Apply diluent **primary antibody**
5 µg/ml RANK: 3000 µl (1: 100 dilution) →
3000 µl required = 30 µl Ab + **2970 µl of diluent**
Volume of ab = total required/ dilution
3000/100 =30µl of Ab + 2970µl of diluent
Volume of diluent required = total required - volume of ab

Add diluted 1° Ab on all **test** slides → 150µl/ slide

Incubate

Overnight at 4°C with PBS

Day 2


Wash Buffer
Wash buffer → shaking (5, 20, 20 mins)
Secondary Antibody
150µl/ slide, calculate for total number of slides
1:200 dilution 20 slides = 3000µl
3000/200 = 15µl 2⁰ Ab + 3000 - 15 = 2985µl of diluent
1: 200 dilution 20 slides = 3000µl
Incubate for 1 hour in incubation box at RM
Wash with PBS for 5, 10, 10 mins → 3 changes
Endogenous Perioxidase Blocking
Incubate slides in 0.3% H2O2 in methanol (prepared by adding 1ml of 30% H2O2 in 100ml of methanol → made fresh) →
Incubate for 10 mins
Wash with PBS 3x 5 mins each
Adivin-biotin-horseradish Peroxidase
Vectorstain Elite ABC used:
Mixture → solution A (120µl) + PBS (3000µl) + solution B (120µl) mix and wait for 30 mins at RT
For 1000µl of PBS → need 0.04*1000 (for every 1000) = solution A (same for solution B)
For 20 slides = 3000µl required (3000*0.04=120 A&B)
Add 150 µl/ slide → incubate for 30 mins at RT in incubation box
Wash with PBS 3x→ 5, 10, 10 mins
3,3' diaminobenidine (DAB) chromogen
Mixture →DAB solution B (90 µl) + Buffer solution A (2910 µl)
DAB solution B (~30µl/ 1 drop) to 1000µl buffer solution A
For 20 slides = 3000µl → 90µl DAB solution B + 2910µl buffer solution A
Add 150 µl/ slide → incubate for 3 mins (3 slides/ batch)
Discard DAB in separate bottle
Wash with dH2O: 3 x 5 mins
Haematoxylin Stain
a. Haematoxylin → 5 secs
b. Running tap water → until clear
c. Scott's water → 1 min
d. Running cold tap water → few secs
e. 50% alcohol → 2 mins
f. 90% alcohol → 2 mins
g. 100% alcohol → 2 mins
h. Xylene → 2 x 5 mins
Mounting
2 drops of DepeX → coverslip

Day 1

E. Rabbit IgG

De-waxing

Xylene: 1-3
Timings: 5 minutes 2x and 10 minutes 1x

Rehydration

Alcohol 100%, 90%, 70%, 50% - 5 minutes each
Distilled water 2x for 2 minutes each

Antigen Retrieval

80°C for 15 mins in Vector H-3300 (Tri-sodium citrate → dilute 1:9)
Working concentration for tri-sodium citrate = 22ml of stock solution + 198ml of dH_2O
Add 220ml of dH_2O in 2 outside containers, 500ml dH_2O in the main pot
After heat retrieval → wash with PBS 2x 5mins
Mark sections with wax pen

Blocking

20% Goat Serum (heat treated) + 1% BSA/ PBS
(Blocking Serum (BS) = ~150 µl/ slide Store on ice.
Total for 20 slides
Incubate at room temperature for 30 minutes (humidified chamber)
Total volume of required: 3000 µl = 150µl x 20 slides
Volume required (600 µl) = 3000 x 0.2
Volume of 1% BSA/PBS required (2400µl) = Total volume (3000 µl) - Total volume of goat serum (600 µl)
Do not rinse off, wipe excess

Immunohistochemical Staining

Ab diluent → 5% goat serum + 1% BSA/ PBS
Mix in 1.5ml eppendorf tube (account for day 1 + 2)
Ab diluent: 5% goat serum + 1% BSA/ PBS in 1.5ml eppendorf tube
Ab diluent required = **7000** µl (350 µl 5% goat serum + 6650 µl 1% BSA/PBS → store ice
Total volume of Ab diluent required = 70**00** µl (for day 1 and 2)
Volume of goat serum required = Total volume of required x 0.05

Apply diluent **Rabbit IgG Control**
5 µg/ml RANKL: 3000 µl (1: 200 dilution) →
3000 µl required = 15 µl Ab + **2985 µl of diluent**
Volume of ab = total required/ dilution
3000/200 =15µl of Ab + 2985µl of diluent
Volume of diluent required = total required - volume of ab

Add diluted 1° Ab on all **test** slides → 150µl/ slide

Incubate

Overnight at 4°C with PBS

Day 2

Wash Buffer	Wash buffer → shaking (5, 20, 20 mins)
Secondary Antibody	150µl/ slide, calculate for total number of slides 1:200 dilution 20 slides = 3000µl 3000/200 = 15µl 2^0 Ab + 3000 - 15 = **2985µl of diluent** 1: 200 dilution 20 slides = 3000µl Incubate for 1 hour in incubation box at RM
	Wash with PBS for 5, 10, 10 mins → 3 changes
Endogenous Perioxidase Blocking	Incubate slides in 0.3% H_2O_2 in methanol (prepared by adding 1ml of 30% H_2O_2 in 100ml of methanol → made fresh) → Incubate for 10 mins Wash with PBS 3x 5 mins each
Advin-biotin-horseradish Peroxidase	Vectorstain Elite ABC used: Mixture → solution A (**120** µl) + PBS (**3000** µl) + solution B (**120** µl) mix and wait for 30 mins at RT For 1000µl of PBS → need 0.04*1000 (for every 1000) = solution A (same for solution B) For 20 slides = 3000µl required (3000*0.04=120 A&B) Add 150 µl/ slide → incubate for 30 mins at RT in incubation box Wash with PBS 3x→ 5, 10, 10 mins
3,3' diaminobenidine (DAB) chromogen	Mixture →DAB solution B (**90** µl) + Buffer solution A (**2910** µl) DAB solution B (~30µl/ 1 drop) to 1000µl buffer solution A For 20 slides = 3000µl → 90µl DAB solution B + 2910µl buffer solution A Add 150 µl/ slide → incubate for 3 mins (3 slides/ batch) Discard DAB in separate bottle Wash with dH_2O: 3 x 5 mins
Haematoxylin Stain	a. Haematoxylin → 5 secs b. Running tap water → until clear c. Scott's water → 1 min d. Running cold tap water → few secs e. 50% alcohol → 2 mins f. 90% alcohol → 2 mins g. 100% alcohol → 2 mins h. Xylene → 2 x 5 mins
Mounting	2 drops of DepeX → coverslip

6. Slide Scoring

Time Points	Cell or Tissue Type	RANK		RANKL		OPG	
		Control	Test	Control	Test	Control	Test
4 Weeks	Osteoclasts						
	Osteoblasts						
	Connective tissue						
Time Points	**Cell or Tissue Type**	**Control**	**Test**	**Control**	**Test**	**Control**	**Test**
8 Weeks	Osteoclasts						
	Osteoblasts						
	Connective tissue						
Time Points	**Cell or Tissue Type**	**Control**	**Test**	**Control**	**Test**	**Control**	**Test**
16 Weeks	Osteoclasts						
	Osteoblasts						
	Connective tissue						

Slide Scoring. For RANK/ RANKL positive staining were scored as + (1-2 positive osteoclasts per section), ++ (3-10 positive osteoclasts per section), +++ (>10 positive osteoclasts per section). ND, no positive staining detected. For RANKL/ OPG positive staining were scored as + (small number of positive cells), ++ (moderate number of positive cells) and +++ (large number of positive cells).

Appendix IV: Gene Analysis

1. Tissue Sample RNA Concentrations

Unique Number	Sheep Number	Group	R P3	Concentration of RNA (ng/µl)	A260	A260/ A280
1.	597	4-week	C	18.4	0.045	1.704
2.	432	4-week	B	60.4	0.198	1.756
3.	586	4-week	C	128.0	0.320	1.899
4.	441	4-week	B	59.6	0.149	1.886
5.	583	4-week	C	11.2	0.028	1.556
6.	428	4-week	B	36.4	0.091	1.820
7.	595	4-week	C	32.8	0.081	1.745
8.	440	8-week	C	19.6	0.047	1.750
9.	589	8-week	B	34.0	0.085	1.771
10.	434	8-week	C	13.2	0.031	1.833
11.	598	8-week	B	14.0	0.035	1.667
12.	426	8-week	C	60.8	0.152	1.831
13.	438	8-week	B	62.8	0.158	1.847
14.	450	8-week	C	67.2	0.169	1.888
15.	430	16-week	C	129.0	0.324	1.878
16.	599	16-week	B	38.4	0.096	1.846
17.	584	16-week	C	47.2	0.119	1.872
18.	585	16-week	B	24.4	0.061	1.848
19.	442	16-week	C	21.2	0.053	1.828
20.	427	16-week	B	19.2	0.048	1.778
21.	425	16-week	C	37.6	0.095	1.843

2. cDNA Synthesis

Step 1: SuperScript IV VILO Master Mix with ezDNase Enzyme reaction setup

	Tube #																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**20**	**21**
Master Mix 10x eDNase Buffer eDNase enzyme	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Template RNA (150ng)	8	4.9	2.3	5	8	8	8	8	8	8	4.9	4.8	4.5	2.3	8	6.3	8	8	8
Nuclease-free water	0	3.1	5.7	3	0	0	0	0	0	0	3.1	3.2	3.5	5.7	0	1.7	0	0	0
	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Measured in uL.

Step 2: cDNA Synthesis

Master Mix	**RT (+) Master Mix**	**X20**
SuperScript IV VILO Master Mix	4	80
SuperScript IV VILO No RT Control	0	0
Nuclease-free water	6	120
	10	200

	Tube #																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**20**	**21**
Reaction mix from Step 1	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Mastermix Step 2	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

3. qPCR Primer Sequence Design Steps

The mRNA coding sequences for sheep (ovis aries), bovine (bos taurus) and human (homo sapiens) were found at the NCBI website www.ncbi.nlm.nih.gov/nucleotide) and extracted in the FASTA format. The four mRNA sequences were then aligned using the clustalo alignment tool at www.ebi.ac.uk/Tools/msa/clustalo/. The alignment file was downloaded. Forward and reverse primers were identified using Primer Premier 5.0 software and the sheep FASTA mRNA sequence. The size of the amplification product size was confined to 5-160bps. The complete primer was reversed on the website www.reverse-complement.com. A BLAST search (www.blast.ncbi.nlm.nih.go/Blast.cgi) for each of the primer sequences was carried out to check they were not complementary to other mRNA sequences.

A. Osteoprotegerin (OPG) TNFRSF11B Ovine

>lcl|XM_004011767.3_cds_XP_004011816.1_1 [gene=TNFRSF11B] [db_xref=GeneID:100037695]
[protein=tumor necrosis factor receptor superfamily member 11B] [protein_id=XP_004011816.1]
[location=185..1393] [gbkey=CDS]

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Note: Yellow highlights selected forward and reverse primer sequences.

B. RANKL (TNFSF11) OVINE

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Green end of coding sequence between the A and G

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Note: Yellow highlights selected forward and reverse primer sequences.

C. COLL1A1 OVINE

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Note: Yellow highlights selected forward and reverse primer sequences.

D. TIMP3 Ovine

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GCACGTGCTCGCCCAGCCACCCCCAGGACGCGTTCTGCAACTCAGACATCGTGATCCGGGCCAAGGTGGT
AGGGAAGAAACTGGTGAAGGAGGGGCCCTTTGGCACACTGGTCTACACCATCAAGCAGATGAAGATGTAC
CGAGGGTTCACCAAGATGCCCCATGTGCAGTACATCCACACGGAAGCCTCTGAAAGTCTCTGTGGCCTTA
AGCTTGAGGTCAACAAGTACCAGTACCTGCTGACAGGCCGTGTCTATGATGGCAAGATGTACACAGGACT
GTGTAACTTCGTGGAGAGGTGGGACCAGCTCACCCTCTCCCAGCGCAAGGGGTTGAACTATCGATATCAC
CTGGGCTGTAACTGCAAGATCAAATCCTGCTACTACCTGCCTTGCTTTGTAACCTCCAAGAATGAGTGTC
TTTGGACCGACATGCTCTCCAATTTCGGCTACCCTGGCTACCAGTCCAAACACTACGCTTGCATCCGGCA
GAAGGGTGGCTACTGTAGCTGGTACCGAGGATGGGCACCCCCGGACAAAAGCATCATCAATGCCACAGAC
CCCTGA

Note: Yellow highlights selected forward and reverse primer sequences.

E. OSTERIX /SP7 Ovine sequence

>lcl|XM_004006300.4_gene_1 [gene=SP7] [db_xref=GeneID:101104389] [location=1..3188] [gbkey=Gene]

GCAGCCGCCGCCGCCACCCGCGGACCCGCAGCCAGGACTCCGGAGTCAGAGCAGGATTCTAGGATCAGAG
CCTGAGTGGGAACAGGAGTGCGGCTGGCCTAGGAGAGGAGCGGAGCCCTCCTCGGACCCGCAGGCGACCC
CTTGCCAGCACTCCCAGTGCCAGACCTCCAGAGAGGAGAGACTCGAGACACCCAGCCCCTTATCCCCCAG
CTCTCTCCATCTGCCTGGCTCCCTGGGACCCGTTCCCCAGCCTCAGGATGGCGTCCTCCCTGCTTGAGGA
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CAGCCTCCAAAACCATGGGGGATGCTTACCCAGCCCCCTTCTCCAGCACCAATGGGCTCCTCTCCCCGCC
AGGCAGCCCTCCAGCACCCACCTCGGGCTATGCCAATGACTACCCTCCCTTTTCCCACTCGTTCCCAGGG
CCCACGGGCACCCAGGACCCTGGGCTCCTAGTGCCCAAGGGGCACAGCTCTTCTGACTGCCTGCCCAGCG
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ACCGGGCCCAGGCAATGCTCCTACCCCTTGGTGGGACATGCATCCTGGGGGCAATTGGCTCGGTGGCGGG
CAGGGCCAGGGTGATGGGCTGCAAGGGACACTGCCTGCAGGCCCTGCTCAGCCTCCATTGAACCCCCAGT
TGCCCACCTACCCGTCCGACTTTGCCCCCCTTAATCCAGCCCCCTATCCAGCTCCCCACCTTCTGCAGCC
AGGGCCCCAGCATGTCTTGCCCCAGGATGTCTATAAACCCAAGGCAGTGGGCAACAGTGGGCAGCTGGAG
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CAGGGCGCTCCTCCTGTGACTGCCCCAACTGCCAGGAGCTGGAGCGCCTGGGCGCTGCCGCAGCCGGGCT
GCGAAAGAAGCCGATCCACAGTTGCCACATCCCAGGCTGTGGCAAGGTGTATGGCAAGGCCTCCCATCTG
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CTGCTCCAAGCGCTTTACCCGAAGCGACCACCTGAGCAAACATCAACGCACCCATGGCGAGCCAGGTCCG
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CGCCCAGACCTTCTGCCTCACCGGCACCCCCAGAAAAAGCCCCTGAAGGCAGTCCGGAGCAGAGCAACCT
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TCCAGATTCCTTGACCCCAGAGCAGGTTCTTCCACTGCCCCTCCTGACACCATGACCCCATTAGGCTAAT
CCCTTGTTGCCGCCTTTAACGGAGCTTGCAGCTGCCATCTTAGACATGCTCTTTGGGGAAGCCCATCTAA
CCCAAGGAGGACATTAGTTTGGAAGTGTCTCTCCTGGAGAAAAGGCGAGGAAGGCTTCCTCTGGGATCAG
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TCATGAGAAAGAAGAATTTATAGATCTAGGAGGACAGAAGTCCCCAAGCTGACTTGGGGTTGCATCCACA
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TGAATGCCTTTCCTGTGACTTTGGATCCTCCTTTTCTACTTCTTGCTCCCTCAAGGCCCCAAGTTAAAGG
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Note: Yellow highlights selected forward and reverse primer sequences.

F. MSX2 Ovine

>lcl|XM_027979998.1_cds_XP_027835799.1_1 [gene=MSX2] [db_xref=GeneID:101107819] [protein=homeobox protein MSX-2] [protein_id=XP_027835799.1] [location=66..869] [gbkey=CDS]

ATGGCTTCTCCGTCCAAAGGCAATGACCTGTTTTCGTCTGATGAGGAGGGCCCAGCGATGGTGGCCGGAC
CGGGCCCGGGGCCTGGGGGCGCCGAAGGGGCAGCGGAGGAACGCCGCGTCAAGGTCTCCAGCCTGCCCTT
CAGTGTGGAGGCGCTCATGTCGGACAAGAAGCCGCCCAAGGAGACGTCCCCGCGGCCAGCCGAAAGCGCC
TCCGCCGGGGCCACCCTGCGGCCGCTTCTGCTGCCTGGCCACGGCGTCCGGGAAGCTCACAGCCCCGGGC
CGCTGGTCAAACCCTTCGAGACCGCCTCGGTCAAGTCGGAAAATTCAGAAGACGGAGCGGCGTGGATGCA
GGAACCGGGCAGATACTCGCCGCCGCCAAGACACATGAGCCCCACCACCTGCACCCTGCGGAAACACAAG
ACCAATCGCAAGCCGCGCACACCCTTTACCACGTCCCAGCTCCTCGCTCTGGAGCGCAAGTTCCGCCAGA
AACAGTACCTCTCCATCGCAGAGCGGGCAGAGTTCTCCAGCTCTCTGAACCTCACAGAGACCCAGGTCAA
AATCTGGTTCCAGAACCGAAGAGCCAAGGCGAAAAGACTGCAGGAGGCAGAACTGGAAAAGCTGAAAATG
GCTGCAAAACCTATGCTGCCCTCCGGCTTCAGCCTCCCTTTCCCCATCAACTCTCCCCTGCAAGCAGCAT
CTCTATACGGTGCATCTTACCCTTTCCATAGACCTGTGCTCCCCATCCCGCCCGTCGGACTCTATGCGAC
GCCAGTGGGATATGGCATGTACCACCTATCCTAA

Note: Yellow highlights selected forward and reverse primer sequences.

G. RANK Species homology

CLUSTAL O(1.2.4) multiple sequence alignment
Human
Bovine
Sheep

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lcl|AF018253.1_cds_AAB86809.1_1             ATGGCCCCGCGCGCCCGGCGGCGCCGCCCGCTGTTCGCGCTGCTGCTGCTCTGCGCGCTG  60
lcl|XM_010819019.3_cds_XP_010817321.1_1     ------------------------------------------------------------  0
lcl|XM_012147022.1_cds_XP_012002412.1_1     ATGGCCCTGCGCGCCCGGCGGCGCCGCCCGCTGCCCGCGCCGCTGGCGCTGCTGGCGCTG  60

lcl|AF018253.1_cds_AAB86809.1_1             CTCGCCCGGCTGCAGGTGGCTTTGCAGATCGCTCCTCCATGTACCAGTGAGAAGCATTAT  120
lcl|XM_010819019.3_cds_XP_010817321.1_1     ------ATGTTCAGGGTGACTTTGCAGATCACCCCTCCATGTACCAGCGAGAGGTATTAC  54
lcl|XM_012147022.1_cds_XP_012002412.1_1     CTCGGCCGGCTGCAGGTGACTTTGCAGATCACCCCTCCGTGTACCAGCGAGAGGCATTAC  120
                                                   * *   **** ********** * ***** ******** **** * ****

lcl|AF018253.1_cds_AAB86809.1_1             GAGCATCTGGGACGGTGCTGTAACAAATGTGAACCAGGAAAGTACATGTCTTCTAAATGC  180
lcl|XM_010819019.3_cds_XP_010817321.1_1     GAGCATCTTGGACAATGCTGTAAGAAATGTGAGCCAGGAACATACATGTCTTCCAAATGC  114
lcl|XM_012147022.1_cds_XP_012002412.1_1     GAGCATCTTGGACAATGCTGTAAGAAATGTGAGCCAGGAACGTACATGTCTTCCAAATGC  180
                                            ******** ****  ******** ******** *******  ********** ******

lcl|AF018253.1_cds_AAB86809.1_1             ACTACTACCTCTGACAGTGTATGTCTGCCCTGTGGCCCGGATGAATACTTGGATAGCTGG  240
lcl|XM_010819019.3_cds_XP_010817321.1_1     ACCACTACGTCTGAGAGCGTCTGTCTGCCCTGTGGCTTGGACGAGTACCTGGACACCTGG  174
lcl|XM_012147022.1_cds_XP_012002412.1_1     ACCACTACCTCTGAGAGCGTCTGTCTGCCCTGCGGCTTGGACGAATACCTGGACACCTGG  240
                                            ** ***** ***** ** ** ********** ***  *** ** *** **** * ****

lcl|AF018253.1_cds_AAB86809.1_1             AATGAAGAAGATAAATGCTTGCTGCATAAAGTTTGTGATACAGGCAAGGCCCTGGTGGCC  300
lcl|XM_010819019.3_cds_XP_010817321.1_1     AATGAAGAAGATAAATGCTTGCTGCACAAAGTCTGCGACCCAGGCAAGGCCCTGAGGGCC  234
lcl|XM_012147022.1_cds_XP_012002412.1_1     AACGAAGAAGATAAATGCTTACTGCACAAAGTCTGCGACCCAGGCAAGGCCCTGAGGGCG  300
                                            ** ***************** ***** ***** ** **  **************  ***
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lcl|AF018253.1_cds_AAB86809.1_1          GTGGTCGCCGGCAACAGCACGACCCCCCGGCGCTGCGCGTGCACGGCTGGGTACCACTGG  360
lcl|XM_010819019.3_cds_XP_010817321.1_1  GTGGAGCCCGGCAACCGGACGGCGCCGCGCCGCTGCGCCTGCACCGCCGGCTACCACTGG  294
lcl|XM_012147022.1_cds_XP_012002412.1_1  GTGGAGCCCGGCAACCGGACGGCGCCGCGCCGCTGCGCCTGCACGGCCGGCTACCACTGG  360
                                         ****   ******** * *** * ** ** ******** ***** ** ** *********

lcl|AF018253.1_cds_AAB86809.1_1          AGCCAGGACTGCGAGTGCTGCCGCCGCAACACCGAGTGCGCGCCGGGCCTGGGCGCCCAG  420
lcl|XM_010819019.3_cds_XP_010817321.1_1  AGCGAGGACTGCCGCTGCTGCACCCGCAACGCCGAGTGCGCGCGCGGCTTCGGCGCCCGG  354
lcl|XM_012147022.1_cds_XP_012002412.1_1  AGCGAGGACTGCCGCTGCTGCAGCCGGAACGCCGAGTGCGCGCGCGGCTTCGGCGCCCGG  420
                                         *** ********   ******  *** *** ************  *** * ******* *

lcl|AF018253.1_cds_AAB86809.1_1          CACCCG**TT**GCAGCTCAACAAGGACACAGTGTGCAAACCTTGCCTTGCAGGCTACTTCTCT  480
lcl|XM_010819019.3_cds_XP_010817321.1_1  CGCCCC**GT**GCAGCTCAACAAGGACACGGTGTGTGAGCCCTGCCCCTCAGGCTACTTCTCC  414
lcl|XM_012147022.1_cds_XP_012002412.1_1  CGCCCC**GT**GCAGCTCAACAAGGACACAGTGTGTGAGCCCTGCCCCGCAGGCTACTTCTCC  480
                                         * ***  ****************** *****  * ** ****   *************

lcl|AF018253.1_cds_AAB86809.1_1          GATGCCTTTTCCTCCACGGACAAATGCAGACCCTGGACCA**AC**TGTACCTTCCTTGGAAAG  540
lcl|XM_010819019.3_cds_XP_010817321.1_1  GACACCGTTTCCGCCACAGAGACGTGCAGACCCTGGACCA**AC**TGTACCATTCTTGGAGGG  474
lcl|XM_012147022.1_cds_XP_012002412.1_1  GACACCGTTTCCGCCACAGAGACGTGCAGACCCTGGACCA**AC**TGTACCATCCTTGGACGG  540
                                         **  ** ***** **** ** *  ********************** * ******  *

lcl|AF018253.1_cds_AAB86809.1_1          AGAGTAGAACATCATGGGACAGAGAAATCCGATGCGGTTTGCAGTTCTTCTCTGCCAGCT  600
lcl|XM_010819019.3_cds_XP_010817321.1_1  ACAGAAGTGCGTCACGGAACCGATAAATTAGATGTGGTTTGTAGTCC---TCTCCCATCT  531
lcl|XM_012147022.1_cds_XP_012002412.1_1  ACAGAAGTGCATCACGGAACTGATAAATCAGATGTGGTTTGTAGTCG---TCCCCTACCT  597
                                         * ** **  * *** ** ** ** ****  **** ****** ***     **  * * **

lcl|AF018253.1_cds_AAB86809.1_1          AGAAAACCACCAAAT**GA**ACCCCATGTTTACTTGCCCGGTTTAATAATTCTGCTTCTCTTC  660
lcl|XM_010819019.3_cds_XP_010817321.1_1  GCAAATCCATCAAAT**GA**ACCGCAGGTTTACTTGCCCAGTTTAATTATTCTGCTGCTCTTC  591
lcl|XM_012147022.1_cds_XP_012002412.1_1  GCAAGTTCACCGAAT**GA**ACCGCAGATTTACTTGCCCAGTCTAATCATCCTGCTCCTCTTC  657
                                           **   ** * ******** **  *********** ** **** ** ***** ******

lcl|AF018253.1_cds_AAB86809.1_1          GCGTCTGTGGCCCTGGTGGCTGCCATCATCTTTGGCGTTTGCTATAGGAAAAAAGGGAAA  720
lcl|XM_010819019.3_cds_XP_010817321.1_1  ATGTCTGTGGCGTTAGTAGCTGCCGTCATCTTCGGTGTTTACTACAGGAAAAAAGGGAAA  651
lcl|XM_012147022.1_cds_XP_012002412.1_1  ACGTCTGTGGCTTTAGTAGCTGCCGTCATCTTTGGTGTTTACTACAGGAAAAAAGGGAAA  717
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                                          *********  * ** ****** ******* ** **** *** ***************

lcl|AF018253.1_cds_AAB86809.1_1           GCACTCACAGCTAATTTGTGGCACTGGATCAATGAGGCTTGTGGCCGCCTAAGTGGAGAT  780
lcl|XM_010819019.3_cds_XP_010817321.1_1   GCACTAACAGCTAATTTGTGGCACTGGGTCAATGAGGCTTGCGGCCGCCTAAATGGAAAT  711
lcl|XM_012147022.1_cds_XP_012002412.1_1   GCACTAACAGCTAATTTGTGGCACTGGGTCAATGAGGCTTGCGGCCGCCTAAATGGAAAT  777
                                          ***** ******************** ************* ********** **** **

lcl|AF018253.1_cds_AAB86809.1_1           AAG---GAGTCCTCAGGTGACAGTTGTGTCAGTACACACACGGCAAACTTTGGTCAGCAG  837
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lcl|XM_012147022.1_cds_XP_012002412.1_1   AAGCAGGAGTCCGCAGGCAACAGTTTCAGCTGCACTCACGTGGAGGCCGCCAGCGCCCGC  837
                                          ***   ****** ****  ******    * * ** ***  **    *    *    *

lcl|AF018253.1_cds_AAB86809.1_1           GGAGCATGTGAAGGTGTCTTACTGCTGACTCTGGAGGAGAAGACATTTCCAGAAGATATG  897
lcl|XM_010819019.3_cds_XP_010817321.1_1   GAAGTCTGTGAGGGTGTCTTCCTGCTGACGCTGGAACAGAAGGTGTTTTCTGAAGACACG  831
lcl|XM_012147022.1_cds_XP_012002412.1_1   GAAGTCTGTGAGGGTGTCTTCCTGCTGACCCTGGAACAGAAGGTGTTTTCTGAAGACACG  897
                                          * **  ***** ******** ******** *****  *****   *** * ***** * *

lcl|AF018253.1_cds_AAB86809.1_1           TGCTACCCAGATCAAGGTGGTGTCTGTCAGGGCACGTGTGTAGGAGGTGGTCCCTACGCA  957
lcl|XM_010819019.3_cds_XP_010817321.1_1   TGCCATCCGGAGGCGGGG------------GGCGCGTGCGCAGCGGCCTGTCCCC-----  874
lcl|XM_012147022.1_cds_XP_012002412.1_1   TGCTGTCCGGAGGCGGGG------------GGCGCGTGCGCAGCGGCCTGTCCCC-----  940
                                          ***   ** **    **             *** **** * **  *   *****

lcl|AF018253.1_cds_AAB86809.1_1           CAAGGCGAAGATGCCAGGATGCTCTCATTGGTCAGCAAGACCGAGATAGAGGAAGACAGC  1017
lcl|XM_010819019.3_cds_XP_010817321.1_1   -TCCGGGGAGACGCCGAGATGCTCTCCTTGGTCAGCGAG------ATCGACGGGGATCCC  927
lcl|XM_012147022.1_cds_XP_012002412.1_1   -CCCGGGGAGACGCCGAGATGCTCTCCTTGGTCAGCGAG------ATCGAGGGGGACCCC  993
                                              * * *** ***  ********* ********* **      ** ** *  **   *

lcl|AF018253.1_cds_AAB86809.1_1           TTCAGACAGATGCCCACAGAAGATGAATACATGGACAGGCCCTCCCAGCCCACAGACCAG  1077
lcl|XM_010819019.3_cds_XP_010817321.1_1   TGCAGGCCAGTGCCCACGGAGGACGAATACACGGACAGGTCCCCCCGGACCGCAGACTCC  987
lcl|XM_012147022.1_cds_XP_012002412.1_1   TGCCGGCCGGTGCCCACGGAGGACGAATACACAGACAGGCCCCCCCGGACCGCAGACTCC  1053
                                          * * * *   ******* ** ** *******  ****** ** *** * ** *****

lcl|AF018253.1_cds_AAB86809.1_1           TTACTGTTCCTCACTGAGCCTGGAAGCAAATCCACACCTCCTTTCTCTGAACCCCTGGAG  1137
lcl|XM_010819019.3_cds_XP_010817321.1_1   GTGGTGGCCCTCACCCCGCCTGGAGGCCGGTC------GCCCTTCCCCGAGCCCCTGGAG  1041
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lcl|XM_012147022.1_cds_XP_012002412.1_1      GTGGTGGTCCTCACCCCGCCTGGAGGCCGGTC------GCCCTTCCCCGAGCCCCTGGAG  1107
                                              *  **  ******   ******* **   **       ** *** * ** *********

lcl|AF018253.1_cds_AAB86809.1_1              GTGGGGGAGAATGACAGTTTAAGCCAGTGCTTCACGGGGACACAGAGCACAGTGGGTTCA  1197
lcl|XM_010819019.3_cds_XP_010817321.1_1      GTGGGCGAGAACGACAGCTTCAGCCAGTGCTTCACGGGGACGGACAGCCTGGAGGGCTCC  1101
lcl|XM_012147022.1_cds_XP_012002412.1_1      GTGGGCGAGAACGACAGCTTCAGCCAGTGCTTCACGGGGACGGACAGCCTGGGGGGCTCC  1167
                                             ***** ***** ***** ** ********************  * ***   * *** **

lcl|AF018253.1_cds_AAB86809.1_1              GAAAGCTGCAACTGCACTGAGCCCCTGTGCAGGACTGATTGGACTCCCATGTCCTCTGAA  1257
lcl|XM_010819019.3_cds_XP_010817321.1_1      GAGAGCCCCCGCCTCCCCATGCCCCCCTGCAGGACTGCGTGGAGGCCCGCGTCCCCCAAG  1161
lcl|XM_012147022.1_cds_XP_012002412.1_1      GAGAGCCCCCGCCTCCCCGCGCCCCCCTGCAGGACTGCGTGGAGGCCCGCGTCCCCCAAG  1227
                                             ** ***  *  *  * *   *****  **********  ****  ***  **** *  *

lcl|AF018253.1_cds_AAB86809.1_1              AACTACTTGCAAAAAGAGGTGGACAGTGGCCATTGCCCGCACTGGGCAGCCAGCCCCAGC  1317
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lcl|XM_012147022.1_cds_XP_012002412.1_1      AAGTCCTTGCACGGAGAAGCGGGGGGCGGCCGGTGCCCGCACTGGGCAGCCGGCGCCC--  1285
                                             ** * ******   *** * **   * ****  ****************** *  **

lcl|AF018253.1_cds_AAB86809.1_1              CCCAACTGGGCAGATGTCTGCACAGGCTGCCGGAACCCTCCTGGGGAGGACTGTGAACCC  1377
lcl|XM_010819019.3_cds_XP_010817321.1_1      ----GCTCTGCAGACGGCTGTGCAGGCTGTGGGGACCTGACTTCGGGGGACCCGGCGCCC  1275
lcl|XM_012147022.1_cds_XP_012002412.1_1      ----GCTCTGCAGTCGGCTGTGCAGGCTGCGGGGACCTGACTGCGGGGGACCCGGCGCCC  1341
                                                  **  ****  * ***  *******  ** ***   **  ** ****   *  ***

lcl|AF018253.1_cds_AAB86809.1_1              CTCGTGGGTTCCCCAAAACGTGGACCCTTGCCCCAGTGCGCCTATGGCATGGGCCTTCCC  1437
lcl|XM_010819019.3_cds_XP_010817321.1_1      GGACTGGAGACCCCCCCGAGTGGACCCTTGCCCCAGTGCGCCTACGGCATGGGCCTCCCG  1335
lcl|XM_012147022.1_cds_XP_012002412.1_1      GGCCTGGAGACCCCCCCGAGTGGACCCTTGCCCCAGTGCGCCTACGGCATGGGCCTCCCG  1401
                                                 ***   ****     ************************ *********** **

lcl|AF018253.1_cds_AAB86809.1_1              CCTGAAGAAGAAGCCAGCAGGACGGAGGCCAGAGACCAGCCCGAGGATGGGGCTGATGGG  1497
lcl|XM_010819019.3_cds_XP_010817321.1_1      CCCGCAGCAGAC------CGGGCCGAGGCGGGGGGCCAGCCCCCGGACGGGGCCGCCGCC  1389
lcl|XM_012147022.1_cds_XP_012002412.1_1      CCCGCAGCAGAG------CGGGCCGAGGCGGGGGGCCAGCCCCCGGACGGGGCCGCCGCC  1455
                                             ** * ** ***        ** * *****  * * *******  *** ***** *  *

lcl|AF018253.1_cds_AAB86809.1_1              AGGCTCCCAAGCTCAGCGAGGGCAGGTGCCGGGTCTGGAAGCTCCCCTGGTGGCCAGTCC  1557
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lcl|XM_010819019.3_cds_XP_010817321.1_1      GAGCTTCCCGGCCCCACGAGGGGGG------------------GCCCCGGTGACCAGCCG  1431
lcl|XM_012147022.1_cds_XP_012002412.1_1      GAGCTTCCCGGCCCCGCGAGGGGGG------------------GCCCCGGTGACCAGCCG  1497
                                               *** **  ** *  ******  *                  *** **** **** *

lcl|AF018253.1_cds_AAB86809.1_1              CCTGCATCTGGAAATGTGACTGGAAACAGTAACTCCACGTTCATCTCCAGCGGGCAGGTG  1617
lcl|XM_010819019.3_cds_XP_010817321.1_1      CCTGCCTCAGGCAGTGTGACTGGAAACAGTAACTCCACGTTTATTTCCAGCGGGCAGGTG  1491
lcl|XM_012147022.1_cds_XP_012002412.1_1      CCTGCCTCAGGCAGTGTGACTGGAAACAGTAACTCCACGTTTATTTCCAGCGGGCAGGTG  1557
                                             ***** ** ** * ************************** ** ***************

lcl|AF018253.1_cds_AAB86809.1_1              ATGAACTTCAAGGGCGACATCATCGTGGTCTACGTCAGCCAGACCTCGCAGGAGGGCGCG  1677
lcl|XM_010819019.3_cds_XP_010817321.1_1      ATGAACTTCAAGGGCGACATCATCGTGGTCTACGTCAGTCAGAACTCGCAGGAGGGCCCG  1551
lcl|XM_012147022.1_cds_XP_012002412.1_1      ATGAACTTCAAGGGCGACATCATCGTGGTCTACGTCAGTCAGAACTCGCAGGAGGGCCCG  1617
                                             ************************************ **** ************* **

lcl|AF018253.1_cds_AAB86809.1_1              GCGGCG------------GCTGCGGAGCCCATGGGCCGCCCGGTGCAGGAGGAGACCCTG  1725
lcl|XM_010819019.3_cds_XP_010817321.1_1      GCGGGGCCGGGCGGCGGCGCGGGGGAGCCGGCGGGACGCCCGGTGCAGGAGGAGAGCTCG  1611
lcl|XM_012147022.1_cds_XP_012002412.1_1      GCGGGGCCGGGCGGCGGCGCGGGGGAGCCGGCGGGACGCCCGGTGCAGGAGGAGAGCCCG  1677
                                             **** *            ** * ******   *** ******************* *  *

lcl|AF018253.1_cds_AAB86809.1_1              GCGCGCCGAGACTCCTTCGCGGGGAACGGCCCGCGCTTCCCGGACCCGTGCGGCGGCCCC  1785
lcl|XM_010819019.3_cds_XP_010817321.1_1      CCGCGCTGCGACTCGTTCGCTGGCCTCGGGCCGCGCTTCCCGGATGCGTGCGCCAGCCTC  1671
lcl|XM_012147022.1_cds_XP_012002412.1_1      CCGAGCCGCGACTCGTTCGCCGGCCTCGGGCCGCGCTTCCCGGACGCGTGCCCCGGCCTC  1737
                                              ** ** * ***** ***** **   *** **************  *****  * *** *

lcl|AF018253.1_cds_AAB86809.1_1              GAGGG---------GCTG---------------CGGGAGCCGGAGAAGGCCTCGAGGCCG  1821
lcl|XM_010819019.3_cds_XP_010817321.1_1      GACGTGGGCCCAGGACTGCAGGAGTGCGGCGCCCCGGAGCCCGACAAGGCCTCGCGGCCG  1731
lcl|XM_012147022.1_cds_XP_012002412.1_1      GGCGTGGGCCCAGGACTGCAGGAGCGCGGCGCCCCGGCGCCCGACAAGGCCTCGCGGCCG  1797
                                             *  *           ***               * ** *** ** ********* *****

lcl|AF018253.1_cds_AAB86809.1_1              GTGCAGGAGCAAGGCGGGGCCAAGGCTTGA------   1851
lcl|XM_010819019.3_cds_XP_010817321.1_1      GTGCAGGAGCAGGGGGCGGCCGAGACGCGGCGCTGA   1767
lcl|XM_012147022.1_cds_XP_012002412.1_1      GTGCAGGAGCAGGGGGCGGCCGAGACGCGGCGCTGA   1833
                                             *********** ** * **** ** *  *
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Note: Yellow highlights selected forward and reverse primer sequences.

6. qPCR Plate layout and Cq and Melting Point Results Data

A. B2M and OPG Plate Layout with Cq Values and Melting Points

Plate Layout with sample number

B2M

OPG

	1	2	3	4	5	6	7	8	9	10	11	12
A	432	432	441	441	428	428	597	597	583	583	595	595
B	589	589	598	598	438	438	440	440	426	426	450	450
C	599	599	585	585	427	427	430	430	584	584	425	425
D	Water	Water										
E	432	432	441	441	428	428	597	597	583	583	595	595
F	589	589	598	598	438	438	440	440	426	426	450	450
G	599	599	585	585	427	427	430	430	584	584	425	425
H	Water	Water										

Cq Values

B2M

OPG

	1	2	3	4	5	6	7	8	9	10	11	12
A	17.5	17.6	17.1	17.3	18.0	18.0	17.0	17.6	19.4	19.5	22.6	23.1
B	20.6	20.9	17.9	18.4	26.4	25.9	18.2	18.6	19.5	19.9	17.9	18.1
C	20.3	20.7	19.1	19.4	18.8	18.8	19.5	19.5	22.7	23.0	21.6	21.7
D	32.8	33.7										
E	25.0	27.9	25.2	26.6	25.5	26.2	28.3	28.2	28.4	28.9	34.1	32.8
F	27.4	27.3	27.8	28.0	24.1	24.0	24.5	24.9	26.6	26.6	24.3	24.8
G	25.4	25.5	28.3	29.0	26.5	26.0	25.7	25.7	29.7	29.9	28.8	29.0
H	40.0	40.0										

Melting Point

B2M

	1	2	3	4	5	6	7	8	9	10	11	12
A	79.1	79.5	79.1	79.3	79.2	79.3	79.0	79.2	79.2	79.3	79.1	79.2
B	79.2	79.0	79.3	79.2	79.4	79.4	79.4	79.3	79.2	79.4	79.4	79.2
C	79.5	79.4	79.6	79.4	79.5	79.5	79.5	79.4	79.7	79.2	79.6	79.6
D	75.4	74.1										

OPG

E	77.0	76.6	77.2	76.8	76.8	76.9	76.9	77.2	76.9	77.2	77.4	76.4
F	77.1	77.0	77.1	77.1	77.3	77.3	77.2	77.1	77.2	77.2	77.2	77.3
G	77.4	77.1	77.2	77.2	77.3	77.4	77.3	77.3	76.7	77.2	77.3	77.1
H	61.1	61.0										

B. RANK and RANKL Plate Layout with Cq Values and Melting Points

Plate Layout with sample number

RANK

	1	2	3	4	5	6	7	8	9	10	11	12
A	432	432	441	441	428	428	597	597	583	583	595	595
B	589	589	598	598	438	438	440	440	426	426	450	450
C	599	599	585	585	427	427	430	430	584	584	425	425
D	Water	Water										

RANKL

E	432	432	441	441	428	428	597	597	583	583	595	595
F	589	589	598	598	438	438	440	440	426	426	450	450
G	599	599	585	585	427	427	430	430	584	584	425	425
H	Water	Water										

Cq Values

		1	2	3	4	5	6	7	8	9	10	11	12
	A	28.9	28.8	26.6	26.6	25.4	24.8	23.7	24.1	25.8	25.7	28.8	28.9
RANK	**B**	24.9	25.3	23.8	23.2	26.4	26.7	21.0	21.2	25.2	25.2	25.3	25.1
	C	24.4	24.5	23.3	23.2	21.8	22.0	24.7	24.7	24.8	24.8	23.5	23.6
	D	33.0	32.4										
	E	29.8	29.1	28.8	28.7	25.5	25.2	25.7	25.8	28.8	29.8	33.2	33.6
RANKL	**F**	28.0	28.5	25.6	26.0	30.9	31.7	22.2	22.3	28.2	29.2	27.3	27.7
	G	26.5	26.9	27.8	27.3	23.7	23.7	26.9	26.8	27.9	28.8	26.7	27.2
	H	34.3	34.7										

Melting Point

		1	2	3	4	5	6	7	8	9	10	11	12
	A	69.5	69.5	69.5	69.5	69.6	69.8	69.9	69.8	69.1	69.7	69.6	69.6
RANK	**B**	69.4	69.7	69.8	69.9	70.8	70.9	69.2	69.1	69.6	69.8	70.0	69.9
	C	69.6	69.7	69.8	70.0	69.7	69.9	69.9	69.9	70.0	75.8	70.0	70.1
	D	69.2	69.0										
	E	74.0	74.0	73.8	73.9	73.8	74.0	74.0	74.2	74.1	73.8	72.8	71.4
RANKL	**F**	74.1	74.1	74.2	74.1	74.8	74.8	74.2	74.2	74.2	74.1	74.2	74.1
	G	74.1	74.1	74.2	74.2	74.1	74.3	74.2	74.3	74.2	74.1	74.2	74.2
	H	71.2	72.3										

C. Col1A1 and TIMP3 Plate Layout with Cq Values and Melting Points

Plate Layout with sample number

		1	2	3	4	5	6	7	8	9	10	11	12
Col1A1	**A**	432	432	441	441	428	428	597	597	583	583	595	595
	B	589	589	598	598	438	438	440	440	426	426	450	450
	C	599	599	585	585	427	427	430	430	584	584	425	425
	D	Water	Water										
TIMP3	**E**	432	432	441	441	428	428	597	597	583	583	595	595
	F	589	589	598	598	438	438	440	440	426	426	450	450
	G	599	599	585	585	427	427	430	430	584	584	425	425
	H	Water	Water										

Cq Values

		1	2	3	4	5	6	7	8	9	10	11	12
Col1A1	**A**	17.8	18.0	17.4	17.5	18.5	18.8	19.4	19.8	19.6	19.7	22.7	22.8
	B	19.4	19.7	19.9	20.1	20.9	21.0	15.8	16.0	18.3	18.4	15.9	16.0
	C	17.6	17.8	21.5	21.5	16.4	16.3	15.9	16.2	20.2	20.0	19.8	20.0
	D	33.6	40.0										
TIMP3	**E**	26.3	25.9	24.2	24.2	22.5	22.6	28.1	28.6	26.9	27.1	31.0	32.5
	F	24.9	25.2	26.5	27.3	28.8	28.2	23.7	23.8	25.6	25.7	35.9	25.4
	G	24.0	24.3	25.5	25.4	23.2	23.3	22.9	22.8	26.8	26.8	25.2	25.2
	H	40.0	40.0										

Melting Point

		1	2	3	4	5	6	7	8	9	10	11	12
Col1A1	**A**	78.2	78.0	78.1	78.0	78.2	78.1	78.1	78.1	77.9	77.8	78.1	78.1
	B	78.3	78.1	78.1	78.1	79.3	79.3	78.2	78.2	78.2	78.2	78.3	78.4
	C	78.3	78.3	78.1	78.2	78.1	78.3	78.3	78.2	78.2	78.2	78.2	78.3
	D	72.3	60.9										
TIMP3	**E**	77.1	77.3	77.3	77.4	77.4	77.4	77.5	77.4	77.3	77.2	77.4	77.4
	F	77.4	77.4	77.4	77.3	78.5	78.4	77.5	77.5	77.5	77.4	69.5	77.4
	G	77.4	77.3	77.4	77.4	77.4	77.4	77.6	77.6	77.5	77.4	77.6	77.6
	H	60.4	61.0										

D. Sp7 and Msx2 Plate Layout with Cq Values and Melting Points

Plate Layout with sample number

		1	2	3	4	5	6	7	8	9	10	11	12
Sp7	**A**	432	432	441	441	428	428	597	597	583	583	595	595
	B	589	589	598	598	438	438	440	440	426	426	450	450
	C	599	599	585	585	427	427	430	430	584	584	425	425
	D	Water	Water										
MSX2	**E**	432	432	441	441	428	428	597	597	583	583	595	595
	F	589	589	598	598	438	438	440	440	426	426	450	450
	G	599	599	585	585	427	427	430	430	584	584	425	425
	H	Water	Water										

Cq Values

Sp7

	1	2	3	4	5	6	7	8	9	10	11	12
A	28.9	28.8	27.4	27.5	29.8	29.0	28.7	31.0	31.0	30.4	33.1	34.7
B	28.6	29.5	30.1	31.2	30.6	31.9	24.4	24.4	29.8	29.3	27.4	27.4
C	28.6	28.8	30.2	31.6	27.5	27.5	27.4	27.4	30.2	30.0	28.7	28.8
D	40.0	40.0										
E	29.7	28.8	27.9	28.3	27.5	27.7	26.0	25.8	26.5	26.9	30.4	32.9
F	27.3	27.6	25.0	25.0	29.8	30.0	22.5	22.5	28.2	27.5	28.5	28.6
G	25.7	25.5	24.9	24.8	24.0	24.1	26.6	26.5	26.7	26.5	25.8	25.8
H	33.9	33.5										

MSX2

Melting Point

Sp7

	1	2	3	4	5	6	7	8	9	10	11	12
A	82.2	82.0	82.1	82.0	82.1	82.4	82.2	82.3	82.0	82.1	82.3	82.3
B	82.2	82.0	82.1	82.1	83.1	83.0	82.3	82.3	81.1	82.1	82.4	82.1
C	82.4	82.3	82.5	82.0	82.2	82.2	82.3	82.3	82.2	82.2	82.5	82.5
D	62.1	60.8										
E	79.3	79.5	79.5	79.5	79.6	79.7	79.6	79.6	79.6	79.6	73.2	73.8
F	79.7	79.5	79.7	79.6	79.4	79.4	79.7	79.8	79.5	79.6	79.7	79.6
G	79.7	79.7	79.6	79.7	79.8	79.7	79.8	79.8	79.8	79.7	79.7	79.7
H	73.9	73.8										

MSX2